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الجامعة الأردنيـــــة
كلية الدراسات العليا

التاريـــخ: / /

نموذج رقم (١٨)
اقرار والتزام بالمعايير الأخلاقية والأمانة العلمية
وقوانين الجامعة الأردنية وأنظمتها وتعليماتها
لطلبة الماجستير

أنا الطالب: آلاء محمد أبو مارة الرقم الجامعي: (٨٠٧٠٢٩٨)

تخصص: ماجستير العلوم الطبية الحيوية الكليـــــة: الطب

عنوان الرسالة:

In vitro selection of anti-FMS like tyrosine kinase-3 (Flt3) aptamers by systemic evolution of ligands by exponential enrichment (SELEX)

اعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة باعداد رسائل الماجستير عندما قمت شخصيا" باعداد رسالتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الرسائل العلمية. كما أنني أعلن بأن رسالتي هذه غير منقولة أو مستلة من رسائل أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة اعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بالغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

توقيع الطالب: آلاء أبو مارة التاريخ: ١١ / ٥ / ٢٠١١

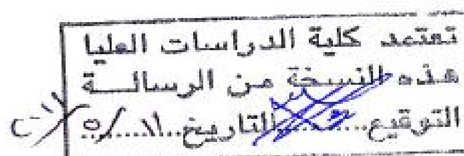
IN VITRO SELECTION OF ANTI- FMS-LIKE TYROSINE KINASE 3 (FLT-3) APTAMERS BY SYSTEMIC EVOLUTION OF LIGANDS BY EXPONENTIAL ENRICHMENT (SELEX)

by
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Dr. Mohammed El-Khateeb, Prof.

This Thesis was Submitted in Partial Fulfillment of the Requirements for the Master's Degree in Medical Laboratory Sciences



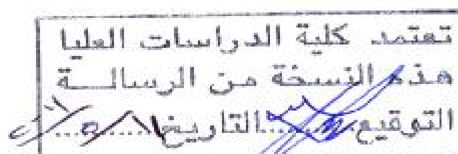
Faculty of Graduate Studies
The University of Jordan

April, 2011

COMMITTEE DECISION

This thesis (IN VITRO SELECTION OF ANTI- FMS-LIKE TYROSINE KINASE 3 (FLT-3) APTAMERS BY SYSTEMIC EVOLUTION OF LIGANDS BY EXPONENTIAL ENRICHMENT (SELEX)) was successfully defended and approved on 27th of April, 2011

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DEDICATION

To the virtuous soul of my father, who had always been proud of achieving this degree;

To my beloved mother, whose overwhelming sympathy wiped off the stress along these years;

To my great brother, Eng. Ahmed, he is the motive beyond this degree;

To my brother Dr. Mohammed, my sisters Dr. Jehan and Dr. Nisreen and their children, for their delicate spirits and continuous support;

To my husband, whose mental and emotional consolidations helped me to get over many problems;

To the light of my life, my son "Muneer";

To my friends, for every thing.

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§ My supervisor, Dr. Said Ismail, who is with his experience, knowledge and great mentality, supports me to do the best,

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§ Mr. Walhan Al-Shaer and Nida'a Ababneh who kindly provided me help and shared with me their experiences, their attitude and discipline are just some of the lessons that I believe I will carry with me for the rest of my life.

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§ Ismail Sami and Yazan Jarrar for every thing they did to help me complete this work,

§ Thanks also go to every member of the microbiology department, for allowing me to use the equipment and materials needed to achieve this work.

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LIST OF ABBREVIATIONS

AMD	**Age-related macular degeneration**
AML	**Acute myeloid leukemia**
BAD	**BCL2 Associated Death Promoter**
bp	**Base pair**
CE	**Capillary electrophoresis**
CREB	**cAMP Response Element Binding protein**
DMSO	**Di-Methyl Sulphoxide**
dNTP	**Deoxy nucleiotides triphosphate**
dsDNA	**Double stranded deoxyribonucleic acid**
EGFR	**Epidermal growth factor receptor**
ELISA	**Enzyme linked immunosorbant assay**
ERK	**Extracellular-signal Regulated Kinase**
4E-BP1	**eukaryotic initiation factor 4E-Binding Protein**
FDA	**Food and drug administration**
FGFR	**Fibroblast growth factor receptor**
FL	**Flt-3 ligand**
Flk-2	**Fetal liver kinase 2**
Flt-3	**Fms- like tyrosine kinase 3**
GAB2	**GRB2 Binding protein**
GRB2	**Growth Factor Receptor-Bound Protein-2**
HA	**Himagglutinin**
HTS	**High throuput screening**
IPTG	**Isopropyl-1-thio-β-D-Galactoside**
JAK2	**Janus kinase 2**
JM	**Juxtamembrane domain**
LS-Rg	**L-selectin receptor globulin**
MALDI-MS	**matrix-assisted laser desorption/ionisation mass spectrometry**
MEK	**MAPK/ERK Kinases**
mTOR	**mammalian Target of Rapamycin**
PCR	**Polymerase chain reaction**
PDGFR	**Platelet derived growth factor receptor**
PDK1	**3-Phosphoinositide-dependent Protein Kinase-1**
PI3K	**Phosphatidylinositol 3-kinase**
PKB	**Protein Kinase-B**
PSMA	**Prostate specific membrane antigen**
PS-Rg	**P-selectin/IgG**
QGRS	**Quadruplex-forming G-rich sequences**
RNA	**Ribonucleic acid**
RNPs	**Ribonucleoproteins**
RTK	**Receptor tyrosine kinase**
SELEX	**Systematic Evolution of Ligands by Exponential Enrichment**
SHCs	**SH2-containing sequence proteins**
SHIP	**SH2-domain-containing Inositol Phosphatase**
SHP2	**SH2-domain-containing protein tyrosine Phosphatase-2**

ssDNA	Single stranded deoxyribonucleic acid
STAT	Signal Transducer and Activators of Transcription
STK-1	Stem cell kinase
TBE	Tris-Borate-EDTA
TE	Tris -EDTA
TKD	Tyrosine kinase domain
TM	Transmembrane domain
VEGF	Vascular endothelial growth factor

IN VITRO SELECTION OF ANTI- FMS-LIKE TYROSINE KINASE 3 (FLT-3) APTAMERS BY SYSTEMIC EVOLUTION OF LIGANDS BY EXPONENTIAL ENRICHMENT (SELEX)

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ABSTRACT

Aptamers are short nucleic acids with specific and complex three dimensional shapes that can be routinely isolated from synthetic combinatorial nucleic acid libraries by in vitro selection process, known as systematic evolution of ligands by exponential enrichment (SELEX). The potential of SELEX/ aptamer technology to provide a new generation of screening tools and novel classes of pharmaceuticals is obvious and rapidly becoming a reality. Tyrosine kinases are enzymes that phosphorylate tyrosine residues by transforming the γ phosphate of ATP to tyrosine residues on selected proteins, including the kinase itself (autophosphorylation), or downstream signaling proteins.

Tyrosine kinases play crucial roles in hematopoiesis, hematopoietic stem cell functions and hematologic malignancies. The Fms- like tyrosine kinase 3 (Flt-3) is a type III receptor tyrosine kinase that is thought to play a key role in hematopoiesis. It confers proliferative and anti-apoptotic effects on normal and leukemic hematopoietic stem cells A ssDNA library consisting of 90 bases sequences was used. This library was constructed to have 45 nucleotides random region flanked by two fixed regions of primer binding sites.

The SELEX method was used to select aptamers against the active tyrosine kinase domain of both Flt-3 enzymes, the wild type and the mutant form D835Y. After eight rounds of selections, aptamer pool was cloned and sequenced. Out of 10^{14} different sequences only one aptamer was obtained. The secondary structures of anti-Flt-3 aptamer were predicted by computer modeling using the Mfold program; moreover, this aptamer was investigated for having G- quadruplex forming motifs comparable to that for the anti-proliferative G- rich sequence of AS1411.

1. Introduction

Cancer is a leading cause of death throughout the world and although many drugs have been developed to treat this disease, the majority of the oncology pharmacopeia causes serious, dose-limiting side effects due to damage to healthy cells. There exists a clear need for better, more specific cancer therapies, not only to increase efficacy against cancer cells but also to decrease the suffering of patients who are subjected to these drug regimens.

Aptamers are short nucleic acids with specific and complex three dimensional shapes characterized by stems, loops, bulges, hairpins, pseudoknots, triplexes or quadruplexes. As they can fold into three dimensional shapes (Kruger, et al., 1982), aptamers can well-fittingly bind to a wide variety of targets, from single molecules to complex target mixtures, or whole organisms (Stoltenburg, et al., 2007).

Aptamers are comparable to monoclonal antibodies in their mechanism of action (Gold et al., 1995; James, 2000). It was initially introduced by two different groups in 1990, and it is based on general nucleic acid and protein separation techniques (Tuerk and Gold, 1990; Ellington and Szostak, 1990).

Aptamers can be routinely isolated from synthetic combinatorial nucleic acid libraries by in vitro selection, known as systematic evolution of ligands by exponential enrichment (SELEX) (Proske, et al., 2005). The SELEX methodology involves a combination of selection of nucleic acid ligands which interact with the target in a desirable manner, for example binding to a protein, with subsequent amplification of those selected nucleic acids (Sampson, 2003). Cycling of the selection amplification procedure is continued until a selected goal is achieved.

The potential of SELEX/ aptamer technology to provide a new generation of screening tools and novel classes of pharmaceuticals is obvious and rapidly becoming a reality (Sampson, 2003).

Tyrosine kinases are enzymes that phosphorylate tyrosine residues by transforming the γ phosphate of ATP to tyrosine residues on protein substrates, on the kinase itself (autophosphorylation), or downstream signaling proteins (Till and HubBard, 2000). They play crucial roles in hematopoiesis, hematopoietic stem cell functions and hematologic malignancies.

The Fms- like tyrosine kinase 3 (Flt-3) is a type III receptor tyrosine kinase that is thought to play a key role in hematopoiesis (Griffith, et al., 2004). It confers proliferative and anti-apoptotic effects on normal and leukemic hematopoietic stem cells (Scholl, et al., 2005).

Certain classes of Flt3 mutations constitutively activate forms of the receptor that are found in significant numbers of patients with acute myelogenous leukemia (AML). The mutations occur either in the activation loop such as the missense point mutation of Asp835Tyr (7% of AML patients) or as internal tandem duplication (ITD) sequences in the juxtamembrane (JM) domain (17-26% of AML patients) (Parcells, et al., 2006; Griffith, et al., 2004; Abu-Duhier, et al., 2001). Accordingly, tyrosine kinases, including Flt3, are considered as potential targets for many newly developed anti-cancer drugs and therapies.

2. Literature review

2.1. Aptamers

The word aptamer is derived from the Latin word "haptein" meaning "to attach to" (Remmele, 2003). Aptamers, an emerging class of therapeutics, are synthetic single stranded DNA, double stranded DNA, RNA or peptides that fold up into unique three dimensional structures, allowing them to bind specifically to other targets ranging from small organic molecules to large proteins (Proske, et al., 2005).

Aptamers range in size from approximately 6 to 40 KDa, produced by a combination of Watson-Crick and noncanonical intramolecular interactions (James, 2000). They are capable of binding to a desired target with high affinity with dissociation constant values (kd) in the low nanomolar to picomolar range, and with high specificity enabling them to distinguish between closely related targets (Proske, et al., 2005). Due to their unique properties, aptamers promise to revolutionize many areas of natural and life sciences ranging from affinity separation to diagnostics and treatment of diseases (Musheev and Krylov, 2006).

The first approved therapeutic aptamer for use in man is an RNA- based molecule (Macugen, Pegaptanib) that is administered locally (intravitreally) to treat age- related macular degeneration (AMD) by targeting vascular endothelial growth factor (VEGF) (Ireson and Kelland, 2006).

Some properties of aptamers make them attractive therapeutic agents; prominent among these are their stability, lack of immunogenicity, small size, the relatively simple techniques and apparatus used for their isolation, and their chemical simplicity (James, 2000; Ireson and Kelland, 2006).

Aptamers are isolated from combinatorial oligonucleotide libraries by a procedure called systematic evolution of ligands by exponential enrichment (SELEX) (James, 2000; Musheev and Krylov, 2006).

2.2. Combinatorial chemistry

Combinatorial chemistry is an important technology for industry as well as biotechnological and pharmaceutical research to discover new materials or molecules with desirable properties, new drugs, and catalysts. It is characterized by the synthesis and simultaneous screening of large libraries of related but structurally distinct compounds to identify and isolate functional molecules. Nucleic acids are very attractive compounds for combinatorial chemistry, since they are able to fold into secondary and tertiary structures and can be amplified by PCR or in vitro transcription easily (Stoltenburg, et al., 2007).

2.3. SELEX

2.3.1 Definition

During the last decade, molecular evolution- based combinatorial approaches have received considerable attention (Wilson and Szostak, 1999). In vitro selection, or SELEX, is a combinatorial chemistry procedure that allows the simultaneous screening and rapid selection of highly diverse pools of different RNA, ssDNA, dsDNA or peptide molecules for a particular feature (Klug and Famulok, 1994; Tuerk and Gold, 1990). Molecular targets in SELEX protocol can be either proteins or small molecules. Strong binders are selected from the initial library by repeated cycles of target binding, selection and amplification (Djordjevic, 2007).

Typically, 6 to 18 iterative cycles of selection and amplification are required to generate suitable aptamers from a starting library (Proske, et al., 2005). The size of the

oligonucleotide library is so large, that in many cases it completely saturates the relevant sequence space (Djordjevic, 2007).

The affinity of the oligonucleotide to their target can be influenced by the stringency of the selection conditions (Djordjevic, 2007). Typically, the stringency is progressively increased in the course of a SELEX process. This can be achieved by reducing the target concentration in later SELEX rounds, or changing the binding and washing conditions (buffer composition, volume, and time) (Marshal and Ellington, 2000).

2.3.2. The SELEX protocol

A partial randomized synthetic oligonucleotide template is constructed containing a random inner region that is flanked on both sides by constant sequences. The random sequence classically consists of 15-75 random positions where all four bases are incorporated with equal probabilities (Ullrich, et al., 2001; Guo, et al., 2008).

The selection procedure using the SELEX protocol involves three main steps of binding, partitioning, and elution. The random DNA/ RNA pool is exposed to the target where binding takes place, and then the bound oligonucleotides are separated from the unbound ones in the partitioning step. These bound oligonucleotides are eluted from the target of interest and amplification of the best fitting molecules will be performed. This selection procedure is reiterated for several rounds until few molecules with the desired binding properties are enriched, (Figure 1). The final pool is cloned into bacterial vector and individual colonies are sequenced. SELEX technology generates aptamers with high binding affinity and specificity (Ullrich, et al., 2001; Guo, et al., 2008).

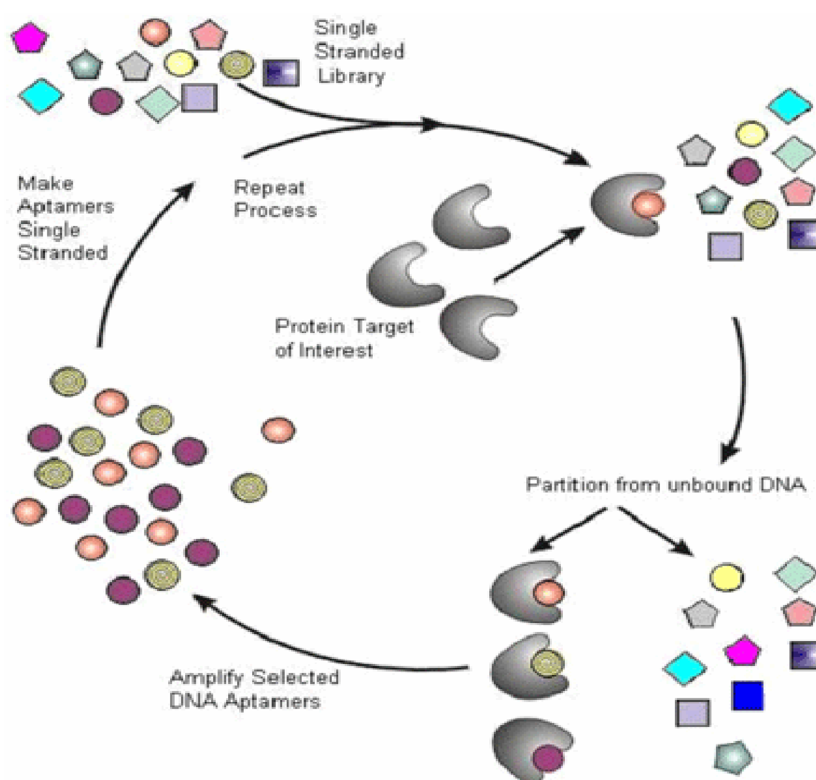


Figure 1. SELEX. (Tuerk and Gold, 1990)

2.3.3. Variations of the SELEX method

Several methods have been attributed to SELEX, regarding the nature of the target, to which aptamers would be generated, and in order to isolate aptamers with improved functionalities. These methods are summarized in Figure (2).

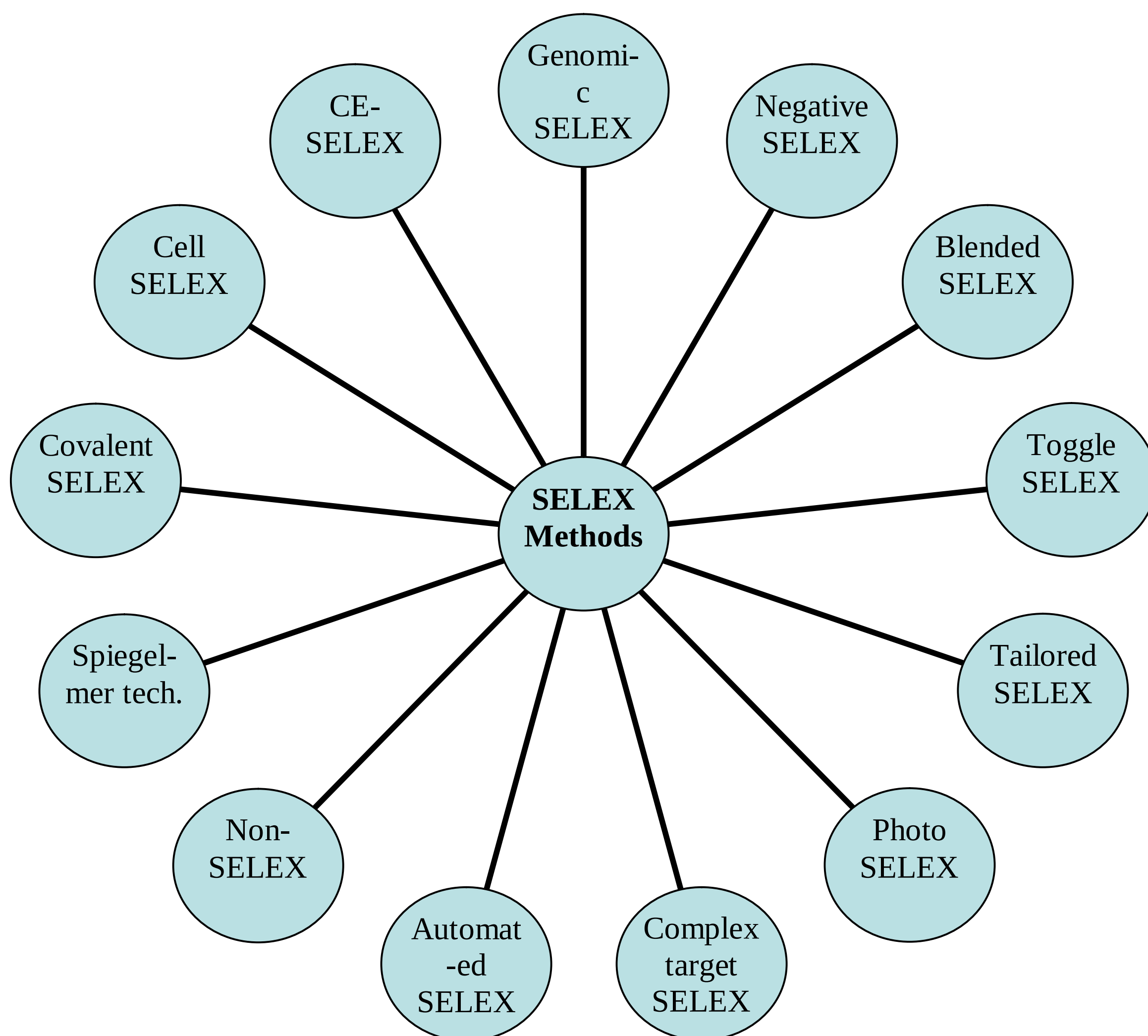


Figure 2. Variations of the SELEX method.

2.3.3.1. Cell- SELEX

Aptamers with high affinity and specificity for cells have been produced successfully, demonstrating that complex targets, including tumor cells and tissues, are compatible with the SELEX process. Cell-SELEX is favored in some special cases, where the clear marker target is unknown. Furthermore, since the target protein domain may be shielded and inaccessible on a cell surface, it reflects a more physiological condition when the protein is displayed on the cell surface rather than isolated as purified protein. Cell-SELEX is easy to handle, fast and reproducible. It makes aptamers an effective tool for

molecular medicine and biomarker discovery, (Figure 3), (Guo, et al., 2008; Morris, et al., 1998).

The cell-SELEX technology plays a crucial role in cancer biology. The generation of aptamers to normal cells enables a comparative strategy to identify differences at a molecular level and to promote the discovery of molecular features of cancer cells; it has raised great expectations for cancer biology, therapy, and regenerative medicine (Guo, et al., 2008).

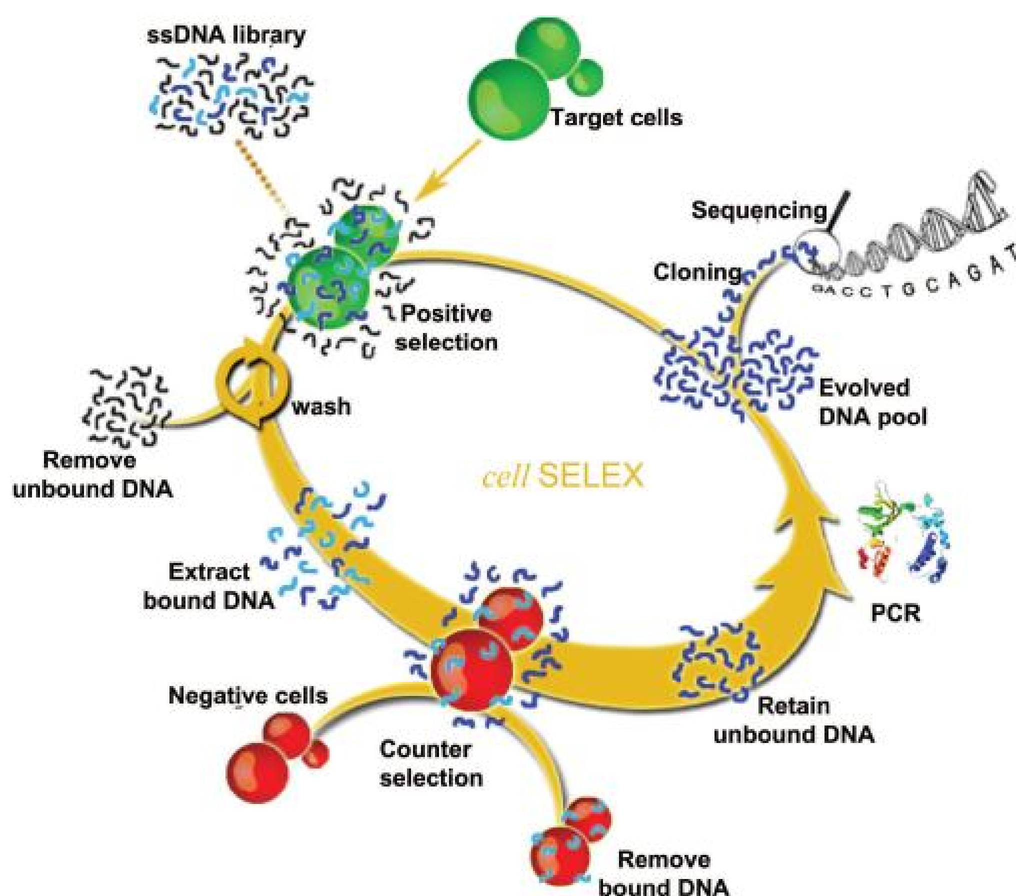


Figure 3. Cell-SELEX procedure (Shangguan, et al., 2006).

Cell-SELEX based aptamers facilitate the development of and simplify the search for molecular probes for diseased cell recognition. For example, Tang et al., (2007) developed an aptamer for the specific recognition of Burkitt's lymphoma cells. Tumor

cell- SELEX also demonstrates the ability of aptamers to identify known tumor markers, such as tenascin-c (Daneils, et al., 2003).

2.3.3.2. Toggle SELEX

Toggle SELEX is used in order to obtain aptamers with different levels of specificity. It relies on switching (toggling) between several protein targets during alternating rounds of selection (Stoltenburg, et al., 2007; Kulbachinskiy, 2007; White, et al., 2001). For example, the library is exposed to the human form of a given protein and in the subsequent round bound to the animal ortholog from the species in which preclinical studies will be conducted (White, et al., 2001). This method should facilitate the isolation of ligands with needed properties for gene therapy and other therapeutic and diagnostic applications (White, et al., 2001).

This method was used to isolate aptamers that bind both human and porcine thrombin during alternating rounds of selection, (Figure 4), (White, et al., 2001).

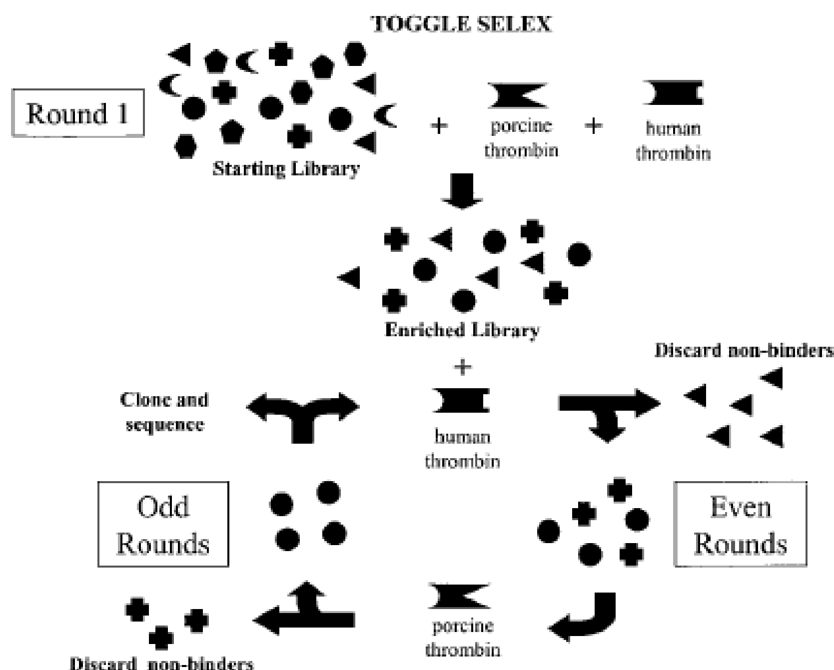


Figure 4. Toggle SELEX (White, et al., 2001).

2.3.3.3. Mirror-image SELEX (Spiegelmer technology)

While aptamers are created from the natural D-nucleotides, which are recognized by the nucleic acid degrading enzymes, a molecule synthesized as the mirror image L-oligonucleotide will be nuclease resistant, as there are no such enzymes in the body capable of interacting with these unnatural molecules. These molecules are termed "spiegelmers" from the German "Spiegel" or mirror. Just like natural D-nucleic acids, single stranded L-oligonucleotides can fold into distinct 3D structures and are thus capable of tightly and specifically binding to a given target (Eulberg and Klussman, 2003).

Spiegelmers are produced using a mirror image SELEX procedure in which D-oligonucleotides are selected from DNA/RNA libraries against synthetic enantiomers of a chosen target, (Figure 5). Being nuclease resistant, inducing minimal immunogenic

response and non-toxic, spiegelmers are ideal candidates for in vivo and in vitro diagnostics; RNA- based and DNA- based spiegelmers have been studied for their applications as in vivo amaging agents (Mairal, et al., 2008).

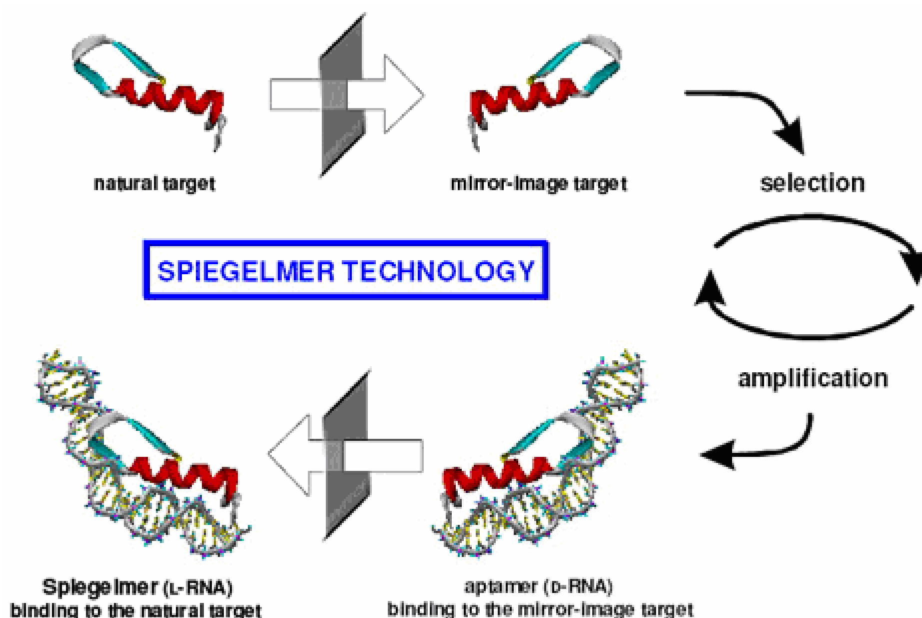


Figure 5. Spiegelmer technology (Purschke, et al., 2003)

2.3.3.4. Non-SELEX

Non-SELEX selection of aptamers is a process which involves repetitive steps of partitioning without intermediate amplification of candidate oligonucleotides, (Figure 6). Efficient partitioning methods should be used with non-SELEX selection in order to enhance the efficiency of the selected aptamers.

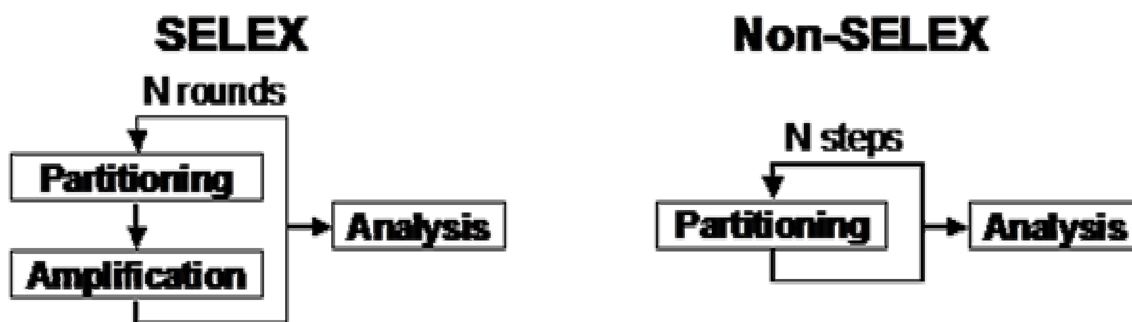


Figure 6. SELEX and non-SELEX procedures (Berezovski, et al., 2006).

This process has a number of advantages over the SELEX procedure. The first advantage is its speed and simplicity: non-SELEX selection takes only about one hour. The second advantage is its ability to accurately determine the abundance of aptamers in the naïve library, which makes it a powerful tool in studies of fundamental properties of DNA libraries. The third and most remarkable advantage of non- SELEX is its potential applicability to nonamplifiable libraries, such as those of DNA- tagged small molecules obtained by DNA- templated synthesis. This feature makes non- SELEX a potentially indispensable tool for drug discovery (Berezovski, et al., 2006).

2.4. Properties of aptamers

2.4.1 High specificity

Aptamers can recognize a large variety of low molecular weight compounds; they are highly discriminating binders (Merger, et al., 2006; Eaton, et al., 1995). Generally aptamers selected against a member of a family of proteins are specific for the target protein versus other family members. For example, an aptamer (14F3'T) to keratinocyte growth factor (KGF), a member of the fibroblast growth factor (FGF) family,

discriminates by over 10,000- fold between KGF and other FGF family members, all of which, notably, are heparin- binding proteins (Pagratis, et al., 1997).

2.4.2 High affinity

The high affinities observed for aptamers likely derive from contacts between aptamer and target protein over an extended surface of both molecules (Tasset, et al., 1997; Jaeger, J., et al., 1998; Haung, et al., 2003).

Aptamers bind to the target with high affinity comparable to those observed with monoclonal antibodies. The aptamer-target binding has an equilibrium dissociation constant (kd) ranging from picomolar to nanomolar concentrations without obvious correlation between the physical and chemical properties of the target and the affinity of binding of aptamer to the target. For example, the anti-L-selectin aptamer has 60 picomolar affinities, while L-selectin has a negative net charge at physiological pH (Jenison, et al., 1998; Watson, et al., 2000). The ability of aptamers to form well- folded stable secondary and tertiary structures also contributes to their binding affinity.

2.4.3. Immunogenecity

Aptamers exhibit little or no immunogenecity, studies in rodents and also in primates and humans have evaluated the immunogenic potential of aptamers. No antigenic response was detected in studies of single- and multiple- dose of an aptamer, which indicates that aptamers have essentially no immunogenic potential (Vater, et al., 2003). Likewise, no antigenic response was observed concerning Macugen for AMD (Eyetech study group, 2002). In preclinical studies, 1000- fold of doses used in animal and human therapeutic applications did not elicit immunologic reactions (Eyetech study group, 2002; White, et al., 2000; Eyetech study group G, 2003).

2.4.4. Toxicity

Aptamers exhibit no or little toxicity when administered to the host as therapeutic agents. The best example is the modified RNA-based aptamer, Macugen. Toxic effects have been reported neither in preclinical studies (Drolet, et al., 2000) nor in phase I trials (Eyetech study group, 2002).

2.4.5. Cost effectiveness

Aptamer production is very cost effective. Aptamers are produced by chemical synthesis rather than biological expression, as in the case of proteins, which makes their production much less expensive compared to the production of antibodies and other therapeutic proteins. The overall expected cost for the production of aptamers will be < 100 US$ /g in the next few years.

2.4.6. Control of selection

Aptamers are in-vitro selected which means that conditions such as pH, ionic strength, temperature and the process of selection is easily controlled to achieve efficient selection (Hianik, et al., 2007; Musheev and Krylov, 2006).

2.4.7. Modifications of aptamers

The first aptamers were discovered within combinatorial libraries of naturally occurring nucleic acids, RNA and DNA (Tuerk and Gold, 1990; Elington and Szostak, 1990). These compositions are quickly degraded in vivo by endogenous nucleases, and consequently are too short-lived for many applications. Subsequent efforts have correspondingly been invested to test a range of modifications that cannot only significantly improve the nuclease resistance of aptamers but which can also confer significant functional improvements.

Considerable stabilization can be achieved by the addition of capping structures at both the 5' and 3' ends of an oligonucleotide and by the incorporation of modifications to

both the ribose and phosphate backbone components (Jellinek, et al., 1995; Kubik, et al., 1997; Lin, et al., 1996; Pagratis, et al., 1997).

Backbone modifications can, in principle, be engineered into aptamer by either direct incorporation into the random sequence pools used to carry out the SELEX process, or alternatively, by post SELEX experimentation.

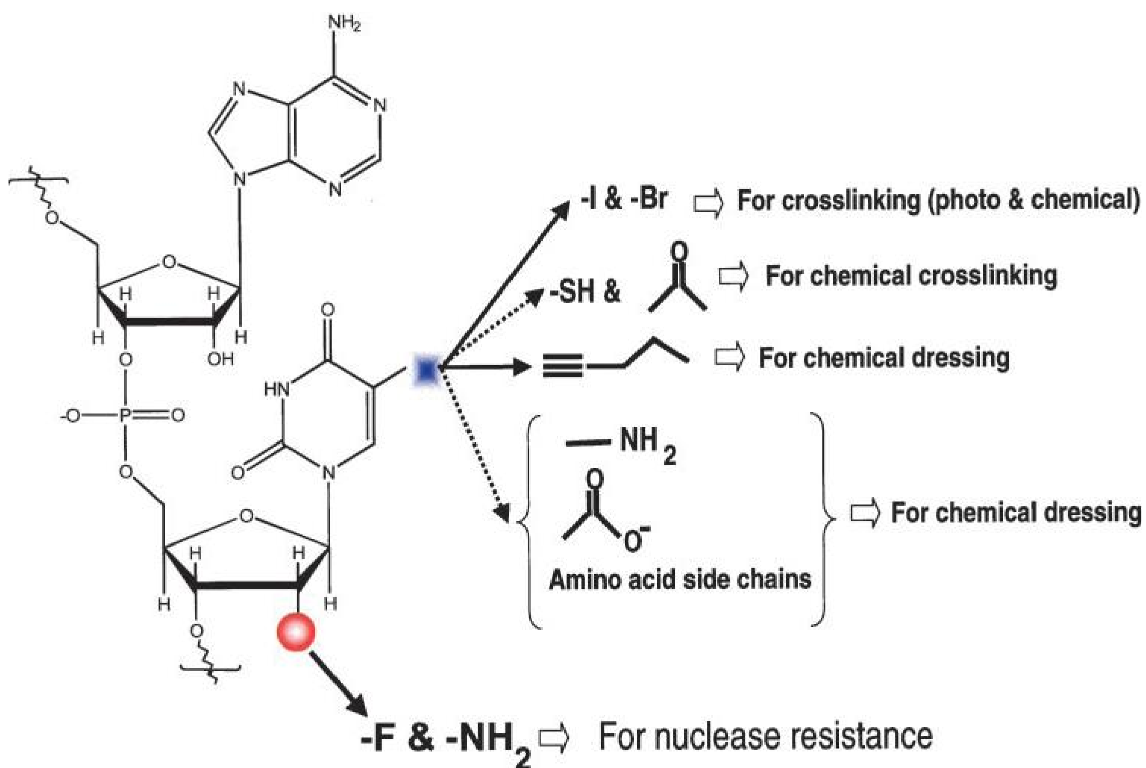


Figure 7. Possible modifications on an oligonucleotide strand to generate modified oligonucleotide libraries for the SELEX process (Jayasena, 1999).

Modification at the 2' position of the sugar confers nuclease stability, whereas various modifications at the C-5 position of the pyrimidines could be used either to attract certain classes of targets or to generate covalent cross-links with targets (Jayasena, 1999).

2.5. Targets for aptamers

2.5.1. Aptamers to small molecules

The RNA world hypothesis led to the belief that aptamers are capable of binding small molecules (Maurel and Hanni, 2005). According to this theory, RNA molecules with catalytic properties, so called ribozymes, should also be able to bind cofactors. Moreover, ribozymes might be regulated allosterically by small cellular components.

2.5.1.1. Aptamers to amino acids

The first aptamers that bound a free amino acid were selected against L-arginine (Connel, et al., 1993). Later, aptamers were selected against variety of amino acids among these are: L-citrulline (Famulok, 1994), aliphatic amino acids and also against L-isoleucine (Majerfeld and Yarus, 1994).

2.5.1.2. Aptamers to carbohydrates

Aptamers to carbohydrates were originally isolated mainly for two different purposes. One was to use an RNA aptamer to the sephadex matrix as a purification tag for RNA or ribonucleoparticles (RNPs) from complex RNA mixtures, the other main purpose was to identify and block specific sugars on cell surfaces. The kd values of carbohydrates aptamers are often higher than those obtained for other macromolecules (Gold, et al., 1995).

Aptamers were isolated to cellulose (Yang, et al., 1998), sialyllactose (Masud, et al., 2004), and an aptamer towards sephadex -100, which consists mainly of repeating units of glucose linked via alpha-1,6 glucosidic bonds was isolated (Srisawat , et al., 2001).

2.5.1.3. Aptamers to natural products

Jenison et al. (1994) selected an RNA aptamer that binds to the alkaloid theophylline with high sensitivity and specificity, with kd values that were remarkably 10,000 times

higher than that to caffeine, which differs from theophylline by a single methyl group at nitrogen atom N7, (Figure 8).

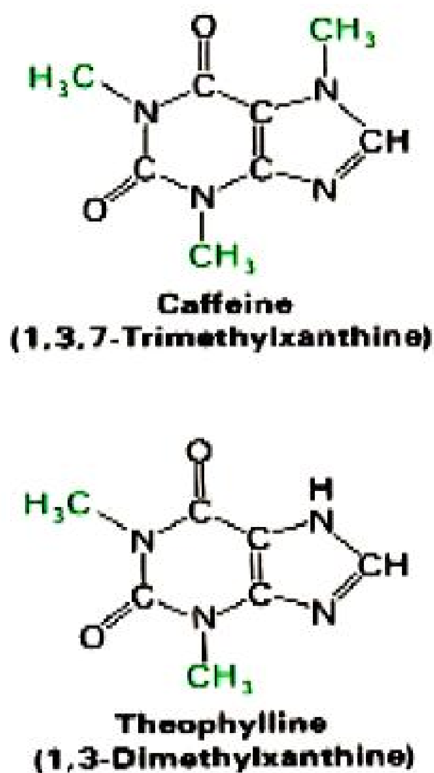


Figure 8. Chemical structures of Theophylline and Caffeine (Jenison et al., 1994).

Also, a DNA aptamer was selected to bind cocaine, this aptamer recognizes only the cocaine and not its metabolites (Stojanovic, et al., 2000).

DNA aptamers were selected to bind the steroid cholic acid (a metabolite of cholesterol) (Kato, et al., 2000).

2.5.2. Aptamers to antibiotics

Many aptamers were isolated against different classes of antibiotics, including aptamers to streptomycin (Klug and Famulok, 1994), aminoglycosides (Wang and Rando, 1995), and to the peptide antibiotic viomycin (Wallis, et al., 1997).

2.5.3. Aptamers to proteins

The properties of aptamers make them an attractive class of molecules that meet and exceed the properties of antibodies (Nimjee, et al., *a*, 2005). These properties are summarized below (Table 1).

Table 1. Properties of Aptamers versus Antibodies (Nimjee, et al., 2005).

Aptamers	Antibodies
Binding affinity in low nanomolar to picomolar range	Binding affinity in low nanomolar to picomolar range
Entire selection is a chemical process carried out in vitro and can therefore target any protein	Selection requires a biological system, therefore difficult to raise antibodies to toxins (not tolerated by animal) or non-immunogenic targets
Can select for ligands under a variety of conditions for in vitro diagnostics	Limited to physiologic conditions for optimizing antibodies for diagnostics
Iterative rounds against known target limits screening processes	Screening monoclonal antibodies time consuming and expensive
Uniform activity regardless of batch synthesis	Activity of antibodies vary from batch to batch
PK parameters can be changed on demand	Difficult to modify PK parameters
Investigator determines target site of protein	Immune system determines target site of protein
Wide variety of chemical modifications to molecule for diverse functions	Limited modifications of molecule
Return to original conformation after temperature insult	Temperature sensitive and undergo irreversible denaturation
Unlimited shelf-life	Limited shelf-life
No evidence of immunogenicity	Significant immunogenicity
Cross-reactive compounds can be isolated utilizing toggle strategy to facilitate pre-clinical studies	No method for isolating cross-reactive compound
Aptamer-specific antidote can be developed to reverse the inhibitory activity of the drug	No rational method to reverse molecules

2.5.4. Aptamers to cytokines and growth factors

Vascular endothelial growth factor receptor (VEGF) is the best characterized factor that is involved in benign and neoplastic angiogenesis. Ruckman and coworkers (1998)

performed 12 rounds of selection and isolated three anti-VEGF aptametrs (Nimjee, et al., *b*, 2005). As mentioned earlier, Pegaptanib (macugen) drug the anti-VEGF RNA aptamer, was the first aptamer-based drug approved by the US Food and Drug Administration (FDA) in 2004 (Eugene, et al., 2006).

Aptamers were also selected to human interferon gamma (Kubik, et al., 1997), angiopoietin-2 (White, et al., 2003), and basic fibroblastic growth factor (Jellinek, et al., 1995).

2.5.5. Aptamers to enzymes

Thrombin is a key regulatory serine protease enzyme in the coagulative cascade. One of the first therapeutic aptamers isolated by SELEX was against human thrombin; ssDNA aptamer was selected after five rounds that exhibited moderate binding affinities for thrombin (Bock, et al., 1992).

Janus kinase 2 (JAK2) is a member of non-receptor tyrosine kinases. It acts as a signal transducer and activator of many transcription pathways and plays important roles in several proliferative disease states such as myeloproliferative disorders. In a study performed at the molecular biology research laboratory (MBRL), medical school, university of Jordan, fourteen rounds of SELEX process were performed to select anti-JAK2 ssDNA aptamers that antagonized and inhibited the kinase activity of this enzyme. The results of this study demonstrated efficient in vitro inhibition to the kinase activity of JAK2 indicating that the selected anti-JAK2 aptamers will be promising in the treatment of many disorders caused by abnormal JAK2 in the future (Alshaer, et al., 2009).

In another example, aptamers have been selected against HIV reverse transcriptase (Tuerk, et al., 1992), where cellular experiments showed a 90-99% reduction in HIV replication (Joshi and Prasad, 2002).

2.5.6. Aptamers to antibodies and immunoglobulins

Many aptamers were selected against a variety of antibodies and immunoglobulins including: anti-insulin receptor antibody MA20 (Doudna, et al., 1995), monoclonal antibody to acetylcholine receptor (Lee and Sullenger, 1997), IgE (Wiegand, et al., 1996), and against cytotoxic T-cell antigen 4 (Santulli-Marotto, 2003).

2.5.7. Aptamers to cell surface receptors and cell adhesion molecules

A cell surface receptor, CD4, is expressed on a variety of immunological cells, most notably helper T cells. It acts to enhance the primary immune response conveyed by the T cell receptor. In 1998, two aptamers were described that were specific to CD4 receptors in the human and rat homologies that bind the receptor with high affinity (Krause, et al., 1998). The selectins, which include L-, E-, and P-selectin, are a family of cell adhesion molecules that are expressed on leukocytes, endothelial cells, and platelets. They are implicated in a number of inflammatory diseases as well as tissue injury and infection. A 2`-fluoropyramidine-modified RNA library was screened by SELEX against P-selectin/IgG (PS-Rg) fusion protein to isolate anti-inflammatory aptamers (Bevilacqua and Nelson, 1993). After twelve rounds of selection, an RNA aptamer with kd value of 18 pmol/L binds to PS-Rg protein on activated human platelets and inhibits both cellular adhesion of PS-Rg to neutrophil and completely blocks neutrophil rolling on activated platelets in vitro (Jenison, et al., 1998).

2.5.8. Aptamers to viral components

New therapeutics were urgently needed for the treatment of pandemic influenza caused by H5N1 influenza virus. Cheng, et al (2008) screened DNA aptamers by SELEX process to target hemagglutinin 1 (HA1) proteins of the H5N1 influenza virus, and after 11 rounds of selection, DNA aptamers that specifically bind to the HA1 protein of H5N1 virus were shown to have antiviral activity in in-vitro studies. These aptamers

would be expected to disrupt virus entry, and thus slow the infection process so the host immune system has time to respond (Cheng, et al., 2008).

Also, in vitro selections were carried out to find aptamers that specifically bind and distinguish the closely related human influenza A virus subtype H3N2. The aptamer displayed over 15-fold-higher affinity to hemagglutinin (HA) compared with the monoclonal antibody, and efficiently inhibited HA-mediated membrane fusion (Gopinath, et al., 2006).

2.6. Applications of aptamers

For a long time nucleic acids were considered mainly as linear carriers of information, whereas most cell functions were ascribed to protein molecules possessing complex three- dimensional structures. However, more examples gradually appeared which showed that single stranded molecules of nucleic acids can carry out numerous intracellular functions (Kulbachinskiy, 2007).

During the past 20 years, aptamers have been developed for various applications such as diagnostics, drug development, target validation and therapeutics (Zhou and Rossi, 2009).

2.6.1. Therapeutic applications of aptamers

Given their small size, ease of synthesis, and low cost, aptamers provide versatile tools for therapeutic applications. As mentioned earlier, the first approved aptamer for use in man is an RNA- based molecule (macugen, pegaptanib) that is administered locally (intravitreally) to treat age- related macular degeneration (AMD) by targeting vascular endothelial growth factor (VEGF) (Ireson and Kelland, 2006).

For therapeutic uses the aptamer has in addition to be a function- blocking compound it has also to directly interrupt the disease process. Aptamers have been reported to act as direct antagonists of the biological function of proteins involved in tumor development by competing with the natural ligands for binding to the same protein domains (Cerchia, et al., 2002).

One of the most advanced aptamers in the cancer setting is AS1411, formerly known as AGRO100, which is being administered systemically in clinical trials, and induces growth inhibition in vitro, and has shown activity against human tumor xenografts in vivo. The mechanism underlying its antiproliferative effects in cancer cells seems to involve initial binding to cell surface nucleolin and internalization, leading to an inhibition of DNA replication (Ireson and Kelland, 2006).

So far, aptamers have been validated as therapeutics in the areas of anti-infectives (Yang, et al., 2007), anti- coagulation, anti-inflammation, anti-angiogenesis, anti-proliferative, and immune therapy (Nimjee, et al., 2005 *a*,*b*). Aptamers could also be used as a "targeting system", for example, binding and carrying an antibiotic agent to the pathogen (Charlton, et al., 1997).

New approaches have been considered to make use of a so-called "antidote" mechanism in order to specifically control the action of an aptamer in vivo. The antidote works by hybridizing the aptamer to a complimentary strand, thus reducing the active structure of the aptamer to an inactive double helix (Merger, et al., 2006).

Rusconi and colleagues developed such a concept and design, utilizing Watson-Crick base pairing between the aptamer and an antidote oligonucleotide to alter the shape of aptamer and then inhibit the binding of aptamer to its target (Figure 9), (Rusconi and Roberts, 2004).

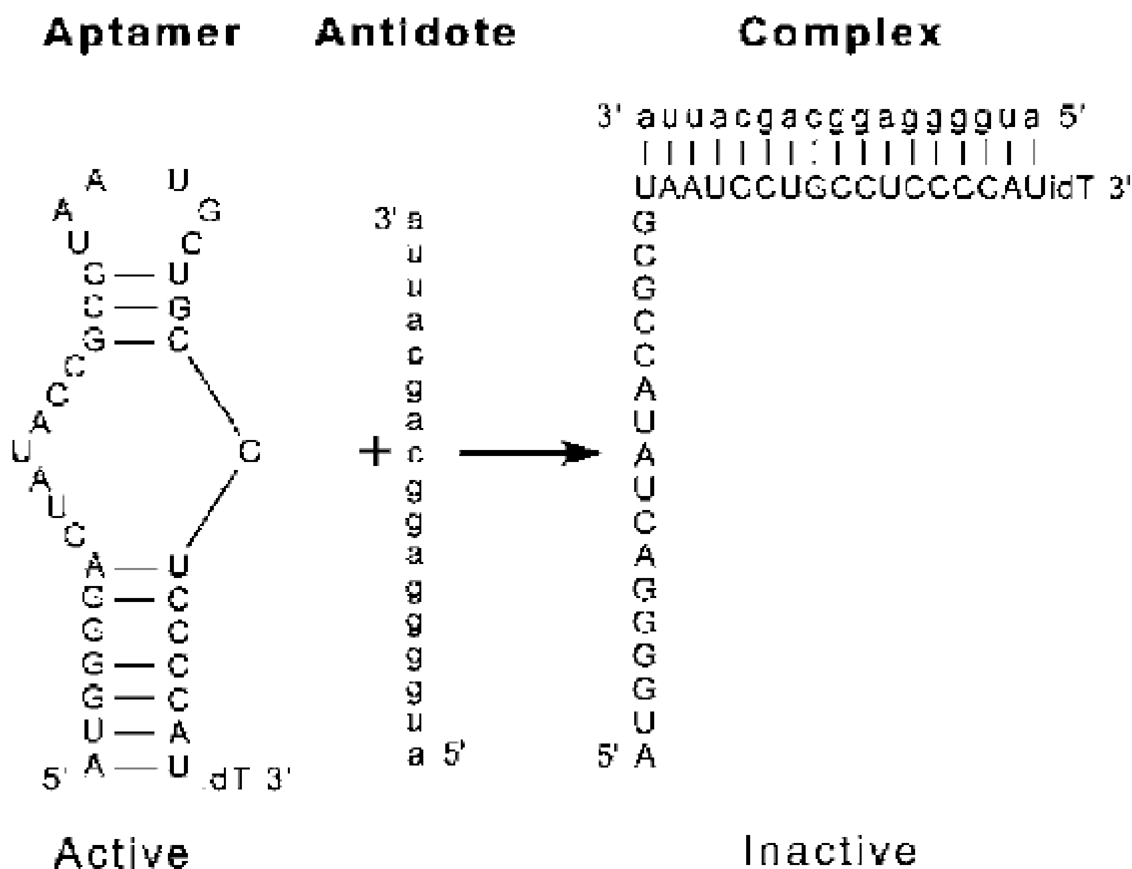


Figure 9. Mechanism of antidote action against aptamers (Rusconi and Robrts, 2004).

2.6.2. Diagnostic applications of aptamers

The high affinity and specificity of aptamers make them ideal diagnostic reagents. Many diagnostic applications rely on ligand-induced conformational changes (Yang, et al., 2007). They inevitably rival antibodies in the field of diagnostics (Proske, et al., 2005). Standard immunochemical methods have been adapted for aptamers, including dot hybridization (Lin, et al., 1996), western blotting (Drolet, et al., 1996), ELISA (Davis, et al., 1996), affinity binding, fluorescence polarization (Gold, et al., 1997), and flow cytometry (Gold, et al., 1997; Davis, et al., 1996; Hicke, et al., 1996).

Somalogic incorporation (Boulder, USA) reported the development of an aptamer chip that can assess approximately 50 different analytes in patient samples (Proske, et al., 2005). Using a so-called proximity- dependent DNA ligation assay, Fredrikson (2002) demonstrated the detection of zeptomolar (10^{-20} molar) concentrations of protein which corresponds to 1,000- fold increase in sensitivity compared to a conventional ELISA (Remmele, 2003; Proske, et al., 2005).

Aptamers have been shown to be useful diagnostic tools for recognizing complex targets such as human RBCs ghosts, for distinguishing differentiated cells from parental cells in carcinoma cell diagnosis, and for application in HIV diagnosis (Wang, et al., 2003; Morris, et al., 1998).

Intriguingly, aptamers against the Alzheimer amyloid peptide were selected; these amyloid aptamers could prove useful in studying and diagnosing the disease (Ylera, et al., 2002).

Lupold et al. (2002) have identified aptamers that bind to prostate cancer cells with low nanomolar affinity through the extracellular portion of the prostate- specific membrane antigen (PSMA) (Lupold, et al., 2002). Standard binding with a radiolabeled aptamer allows quantitation of proteins. An I^{123}- labeled aptamer to thrombin has been used for in vivo detection of clots (Dougan, et al., 1997).

2.6.3. Analytical applications of aptamers

The versatility of aptamers is reflected by the fact that there are few areas of research that aptamers cannot be applied.

2.6.3.1. Aptamers as biosensors

There is a high demand for convenient methodologies for detecting and measuring the levels of specific proteins in biological and environmental samples as their detection,

identification and quantification can be very complex, expensive and time consuming (Styrelitz, et al., 2008).

Aptamers are well-suited to application in biosensors to specifically detect a large variety of molecules like proteins, metabolites, amino acids, nucleotides, etc… (Remmele, 2003). Coupling the aptamer to an appropriate detection system offers the possibility of sensing analytes in solution (Bang, et al., 2005).

A biosensor is a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is here the aptamer, which is in direct spatial contact with a transducer element (Mairal, et al., 2008).

One of the first examples of an optical aptasensor was reported for the detection of L-adenosine by immobilizing a biotinylated RNA aptamer on streptavidin- derivatized optical fibers. FITC- labeled L-adenosine was detected with a 1,700-fold discrimination with respect to D-adenosine by total internal reflection fluorescence measurements (Mairal, et al., 2008; Kleinjung, et al., 1998).

A beacon aptamer-based biosensor for the detection of thrombin was developed using electrochemical transduction method. Gold surface was modified with a beacon aptamer covalently linked at 5' terminus with a linker containing a primary aliphatic amine. Methylene blue was intercalated into the beacon sequence and used as an electrochemical marker. When the beacon aptamer immobilized on gold surface encounters thrombin, the hairpin forming beacon aptamer is conformationally changed to release the intercalated methylene blue, resulting a decrease in electrical current intensity in voltamogram (Bang, et al., 2005).

Biosensors have been developed for more than 25 years now. However, they have not entered the market as much as expected, which is due to several reasons. One reason is

the instability of the biological recognition element of the biosensor (e.g. enzyme, antibodies or cells). The use of biologically stable aptamers as biorecognition elements make them more versatile as biosensors (Styrelitz, et al., 2008; O'Sullivan, 2002).

2.6.3.2. Chromatography

Aptamer affinity chromatography has been used to achieve chromatographic separation and concentration by using aptamers as affinity ligands. (Zhao and Chris, 2009). Aptamers represent a great promise as molecular recognition tools for their incorporation into analytical devices and they can be used as immobilized ligands in separation technologies as in the field of affinity chromatography (Clark and Remcho, 2002). For example, an aptamer specific for L-selectin has been used to purify human L-selectin receptor globulin (LS-Rg) fusion protein produced in Chinese hamster ovary cells transfected to express the protein. The 5`-biotinylated aptamer was fixed to a streptavidin-modified resin and then the resin was packed into a chromatographic column. In the first purification step, the aptamer-based column yielded a 1,500-fold purification with 83% recovery. As a negative control, a column was prepared with DNA having a scramble sequence with respect to the specific aptamer and purified LS-Rg was applied to this column. The new sequence failed to bind detectable levels of LS-Rg demonstrating the high selectivity of the column (Roming, et al, 1999).

2.6.4. Aptamers for biomarker discovery

Biomarkers are molecular signatures associated with the quantity, state, or localization of biomolecules in the cells. They are measurable indicators of cellular states, which are used to screen for diseases and guide medical treatments (Etzioni, 2003). Biomarkers can also serve as surrogate end points in pharmaceutical drug trials, despite the intensified academic and commercial interests and significant investments. Relatively,

few biomarkers are used in clinical practice, and the rate of their introduction is falling (Gutman and Kessler, 2006). The two major reasons for this are the absence of efficient methods for biomarker discovery and the difficulties in implementing practical biomarker-based assays in the clinical environment (Anderson, 2005). Conventional methods for biomarker discovery include Western blotting, mRNA screening using quantitative PCR or hybridization arrays, and two-dimensional gel electrophoresis combined with mass spectrometry (Meyer and Stuhler, 2007; Kelly and Ghosh, 2005; Lescuyer, et al., 2007). These methods share an important common limitation: they are prone to false positive and false negative results. These limitations could be overcome by the technology of aptamer-facilitated biomarker discovery (AptaBiD) (Berezovski, et al., 2008). AptaBiD is based on multiround generation of aptamers for differential molecular targets on the cells which facilitates “exponential detection” of biomarkers. The multiple rounds suppress stochastic variations in cell populations and unintended differences in cell processing, thus, reducing the false positive results. The “exponential detection” of biomarkers allows for sensing minor differences in molecular targets between two cell populations if the differences persist from round to round (Berezovski, et al., 2008).

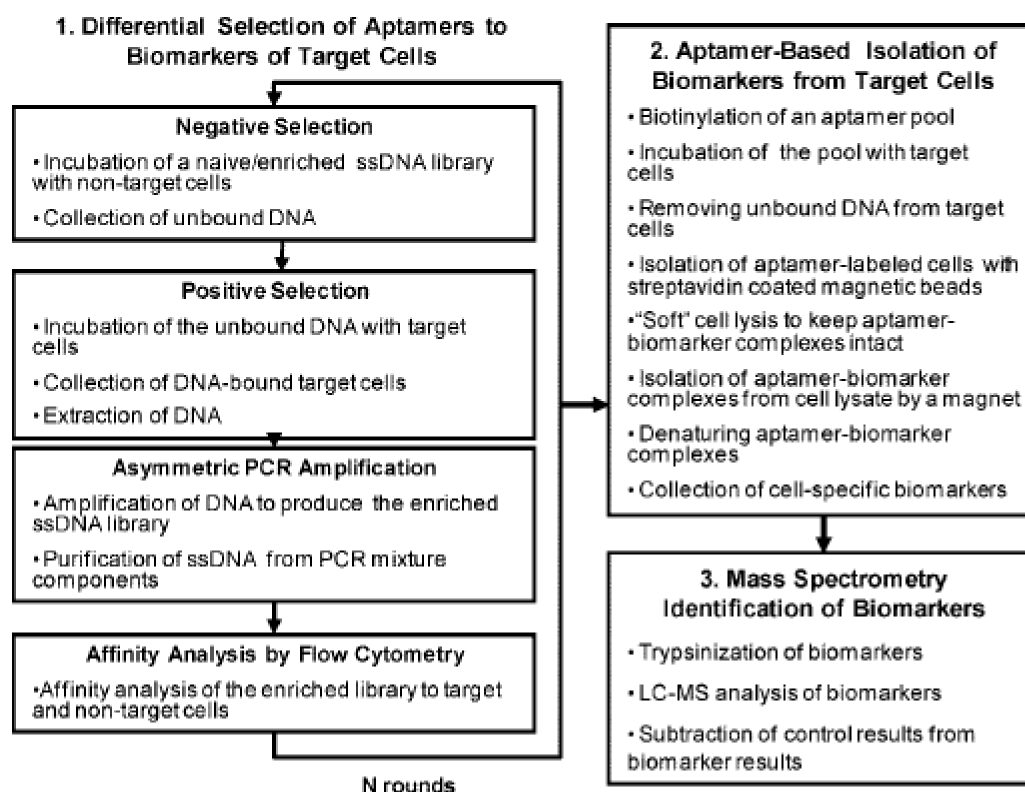


Figure 10. Aptamer-facilitated biomarker discovery (AptaBiD) for cells (Berezovski, et al., 2008).

2.6.5. Aptamers as delivery vehicles

The combination of targeted delivery and controlled drug release (Langer, 1998; Langer, 2001) are potentially desirable properties when treating oncologic diseases where it is desirable that a cytotoxic dose of the drug is delivered to cancer cells over an extended period of time without killing the surrounding noncancerous tissue. Critical to achieving this goal is the engineering of specialized vehicles that encapsulate chemotherapeutic drugs for controlled release, and the targeting of these vehicles to cancer cells with ligands that recognize tumor-specific or tumor-associated antigens (Farokhzad, et al., 2004).

Aptamers targeting cell surface proteins are being explored as promising delivery vehicles to target a distinct disease or tissue in a cell-type-specific manner (Zhou and Rossi, 2009).

Considering the many favorable characteristics of aptamers, including small size, lack of immunogenicity, and ease of isolation, which together have resulted in their rapid progress into clinical trials , it is interesting to examine these molecules for targeted delivery of controlled release polymer drug delivery vehicles. As proof of concept RNA aptamers that bind to the prostate-specific membrane antigen (PSMA) were used to deliver functional siRNA molecules to cells that express PSMA antigen by generating aptamer: streptavidin: siRNA conjugate. The delivery using these aptamer conjugates was found to be efficient and specific for the cells expressing the PSMA (Chu, et al., 2006; Farokhzad, et al., 2004).

The aptamer-based delivery of siRNAs can often enhance the therapeutic efficacy (since siRNA is easily degraded in serum) and reduce the unwanted off-target effects of siRNAs. In particular, for RNA interference-based therapeutics, aptamers represent an efficient agent for cell type-specific, systemic delivery of these oligonucleotides (Zhou and Rossi, 2010).

2.7. G- quadruplex forming sequences

The quadruplex structures formed by guanine-rich nucleic acid sequences have received significant attention because of growing evidence for their role in important biological processes and as therapeutic targets (Simonsson, 2001; Simonsson, et al., 1998; Davis, 2004; Sharma, et al., 2005; Kelland, 2005).

The G- quadruplex structure also known as a G-quartet, is formed by repeated folding of either the single polynucleotide or by association of two or four molecules. The

structure consists of stacked G-tetrads, which are square co-planner arrays of four guanine bases each, Figure (11). G-quadruplex is stabilized with cyclic Hoogsteen hydrogen bonding between the four guanines within each tetrad (Gellert, M., et al., 1962).

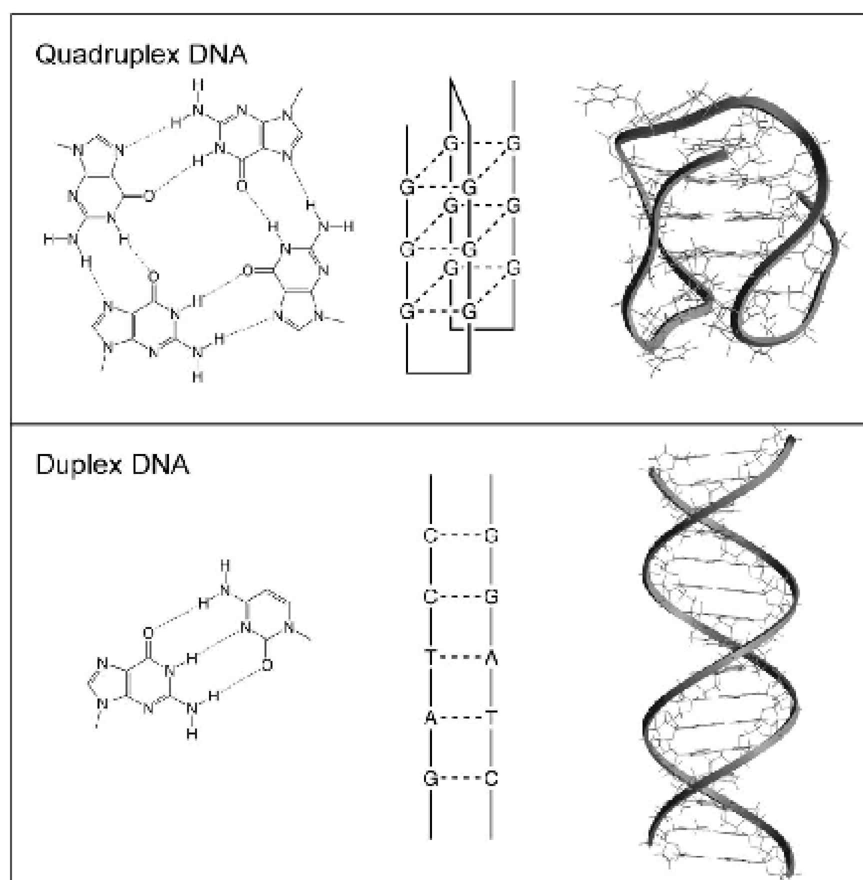


Figure (11). Structures of Quadruplex and Duplex DNA (Bates, et al., 2009)

Properties and functions of G- quadruplex forming sequences

G- quadruplex forming sequences are known to be more functional, with increased nuclease resistance. The role of the quadruplex structure in the enhanced nuclease resistance was confirmed by Bishop, et al., (1996) by the finding that a single G→A mutation that was expected to disrupt quadruplex formation resulted in a dramatically

decreased half-life in serum. Similar findings regarding the enhanced serum stability of quadruplex-forming phosphodiester oligonucleotides were also reported by Dapic, et al (2002). In that paper, a 5′-^{32}P-labeled analogs of GRO29A (a nucleolin-binding antiproliferative oligonucleotide) with backbones consisting of phosphorothioate DNA, phosphodiester DNA (with or without 3′-amine modification), or 2′-O-methyl RNA. All of these analogs were similarly resistant to degradation when placed in serum-containing medium. Interestingly, the unmodified phosphodiester DNA version of GRO29A was completely intact after 5 days in serum-containing medium, whereas for a random sequence of phosphodiester DNA, no full-length oligonucleotide remained after one hour (Dapic, et al., 2002).

G-quadruplex DNA also has shown enhanced cellular uptake (Choi, et al, 2009). Bishop et al., (1996) had found that quadruplex forming anti-HIV oligonucleotides were efficiently internalized by HeLa cervical carcinoma cells, reaching an intracellular concentration that was 8 to 10-fold higher than that in the medium. In contrast, the cellular uptake of non-quadruplex sequences was negligible. In another study, addition of a G10 sequence to the 3′-end of an antisense oligodeoxynucleotide increased its uptake in J774E macrophage cells by 10-fold compared to the unmodified or 3′-C10 oligonucleotide (Prasad, et al., 1999).

In addition, G-quadruplex DNA has been suggested to regulate DNA replication in retinoblastoma susceptibility gene (Rb) region (Xu and Sugiyama, 2006), and control cellular proliferation at telomeric level and by transcriptional regulation of oncogenes (Simonsson, et al., 1998). G-quadruplexes are also being eyed as potential antimicrobial agents due to their ability to transport monovalent anions (Kaucher, et al., 2006).

The effect of G- rich sequences on the proliferation of leukemic cells

Many common types of cancer, especially in their advanced stages, do not respond well to traditional chemotherapy agents or may acquire resistance to therapy. Novel agents that work by mechanisms that are different from existing therapies, particularly those that are able to target multiple pathways important for cancer cell survival, may therefore be useful in the treatment of advanced cancer.

Cancer selective antiproliferative activity is a common characteristic of G-rich DNA oligonucleotide (Choi E. W., et al, 2009). Oligonucleotides containing guanosine residues, G-rich oligonucleotides (GROs), can inhibit the proliferation of several human tumor cell lines in vitro (Bates, et al., 1999; Weerasinghe, et al., 2007; Schwartz, et al., 2008). GROs are also capable of inhibiting DNA replication and inducing cell cycle arrest predominantly at S phase (Bates, et al., 1999; Dapic, et al., 2002; Schwartz, et al., 2008; Tsolou, et al., 2008). Therefore, GROs have been considered as promising therapeutic agents for malignant tumors. The mechanism of GROs' antiproliferation activity is probably due to the formation of stable quadruplex structure and G-quartets (Burgess, et al., 1995; Benimetskaya, et al., 1997; Wang, et al., 1998; Gilbert and Feigon, 1999; Dapic, et al., 2002, 2003; Zhang, et al., 2003). These structures interact with a specific cellular protein, nucleolin (Bates et al., 1999; Xu et al., 2001; Girvan et al., 2006; Goodchild et al., 2007; Teng et al., 2007), leading to the inhibition of cell proliferation. Nucleolin has been implicated in the regulation of cell proliferation and growth (Mi, et al., 2003; Otake, et al., 2007). In response to stress, nucleolin expression is increased and P53 is activated. Activated P53 stimulates the formation of nucleolin-P53 complex, which participates in transient inhibition of DNA replication and initiation of DNA repair (Daniely, et al., 2002).

It has been reported that GROs have antiproliferative activity against a variety of cancer cell lines (Bates, et al., 1999; Xu, et al., 2001; Dapic, et al., 2002, 2003). However, the inhibitory effect of GRO varies in different human cancer cell lines, especially in cell lines with or without P53 expression (such as U937 and A549 cells). The biological activity of GROs might result from their binding to nucleolin. However, the relationship between the effects of GRO, nucleolin, and P53 is unknown.

Initial studies of GROs were carried out using GRO29A, a 3′-modified 29-nucleotide phosphodiester oligodeoxynucleotide (Bates, et al., 1999; Xu, et al., 2001; Dapic, et al., 2002, 2003). It was found that the 3′-modification was not required for either nuclease resistance or activity (Bates, et al., 1999, Dapic, et al., 2002) and that the 5′-terminus "tail" (5′-TTT) of GRO29A was not essential for activity (Xu, et al., 2001).

This guanine rich oligonucleotide (GRO) has the sequence of

5-GGTGGTGGTGGTTGTGGTGGTGGTGG-3 and was studied for its effect on human monocytic leukemia cell line U937 cells (Lei Z., et al., 2011). It was noticed that this GRO can inhibit proliferation of U937 cells and induce cell cycle arrest at S phase (Lei Z., et al., 2011).

2.8. Tyrosine kinases

2.8.1. Definition

Protein tyrosine kinases are proteins that catalyze the transfer of gamma phosphate group from ATP to hydroxyl group of tyrosine residues in polypeptides. The human genome contains about ninety tyrosine kinases and forty three tyrosine kinase-like genes (Krause and Van Etten, 2005; Reilly, 2003).

Tryrosine kinases are divided into two main classes; receptor tyrosine kinases (RTK), which are transmembrane proteins with a ligand- binding extracellular domain and a

catalytic intracellular kinase domain. There are 58 types of RTKs divided further into 16 subclasses. Examples for such kinases are: epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), platelet derived growth factor receptor (PDGFR), insulin receptor (IR), and Fms- like tyrosine kinase 3 (Flt-3). The other class is the nonreceptor (cytoplasmic) tyrosine kinases that lack transmembrane domain, they are found in the cytosol, nucleus, and the inner surface of the plasma membrane. They are divided into 32 classes. Examples include: SRC, ABL, and JAK. (Krause and Van Etten, 2005; Cowan- Jacob, 2006).

The kinase domain of all tyrosine kinases has a bipolar structure, with an N-terminal lobe that binds ATP and magnesium, a C-terminal lobe containing an activation loop, and a cleft between the lobes to which polypeptide substrates bind (Krause and Van Etten, 2005). The structures of the tyrosine kinases in the active state are all very similar, despite the fact that they have different substrate specificities and different mechanism for control (Cowan- Jacob, 2006).

Tyrosine kinases play a key role in the regulation of signal transduction during a plethora of eukaryotic cell functions, including cell activation, cell-cycle progression, cytoskeletal rearrangement, cell movement, differentiation, apoptosis and metabolic homeostasis (Reilly, 2003; Cowan- Jacob, 2006). With such critical role in signal transduction, the tight regulation of kinase activity is crucial (Cowan- Jacob, 2006).

Ligand binding to the receptor tyrosine kinase initiates a cascade of events, including receptor homodimerization, activation of intrinsic kinase activity, intramolecular tyrosine trans-phosphorylation, association with signal- transducing proteins and phosphorylation of substrates (Scheijen and Griffin, 2002).

Tyrosine kinases are implicated in several steps of neoplastic development and progression (Paul and Mukhopadhyay, 2004), hence the identification and development

of therapeutic agents for disease states that are linked to abnormal activation of tyrosine kinases due to enhanced expression, mutation or autocrine stimulation leading to abnormal downstream oncogenic signaling have taken a central stage as potent targets for cancer therapy (Workman, 2003; Sawyers, 2002).

Class III receptor tyrosine kinase share sequence homology and a similar overall structure with five immunoglobulin- like repeats in the extracellular domain, a single transmembrane domain (TM), a juxtamembrane domain (JM), two intracellular tyrosine kinase domains (TK1 and TK2) divided by a kinase insert domain (KI), and a C-terminal domain (Figure 12) (Yarden and Ullrich, 1988). Mutations to this group of receptors have been linked recently to the pathogenesis of myeloid malignancies (Reilly, 2002).

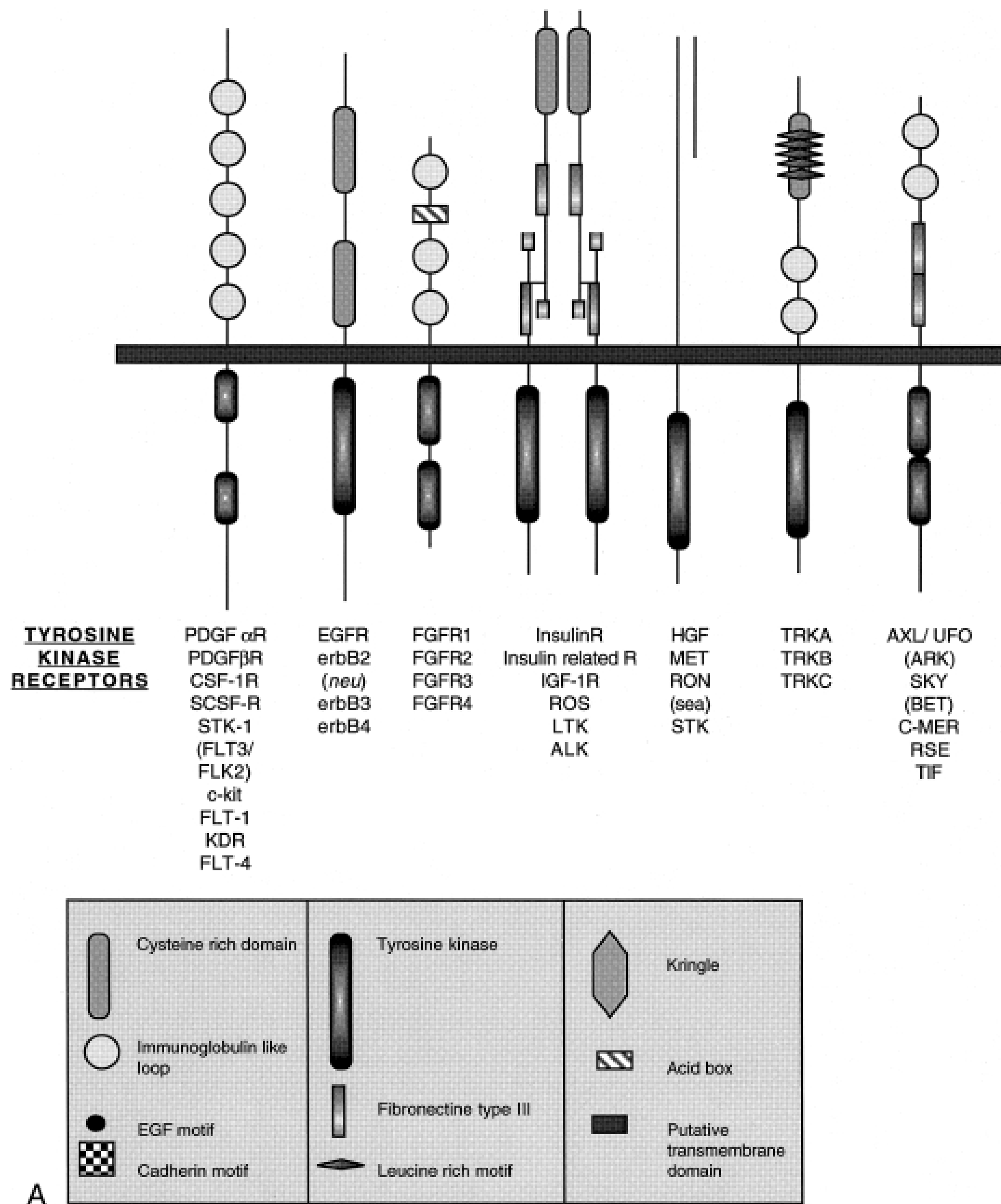


Figure 12. Structure of tyrosine kinase receptors (Yarden and Ullrich, 1988).

2.8.2. Fms- like tyrosine kinase receptor 3 (Flt-3)

2.8.2.1. Molecular biology and structure of Flt3

Also known as CD135, fetal liver kinase-2 (Flk-2), and human stem cell kinase 1 (STK1), is a 993 amino acid class III RTK encoded by a 24 exon gene located on chromosome 13 (13q12). Flt-3 is a transmembrane 160 KDa protein that plays a crucial role in normal hematopoiesis (Doepfner, et al., 2007). It confers proliferation and anti-apoptotic effects on normal and leukemic hematopoietic stem cells (Scholl, et al., 2005). In normal bone marrow *FLT-3* is expressed at high levels in early progenitor cells but is also expressed at abundant levels in a high proportion of cases of acute leukemia, suggesting that Flt-3 may play a role in survival or proliferation of leukemic blasts (Chalandon, et al., 2005).

FLT-3 gene is the most frequently mutated gene in acute myeloid leukemia AML (one third of patients), and confers a poor clinical prognosis (Doepfner, et al., 2007; Parcells, et al., 2006).

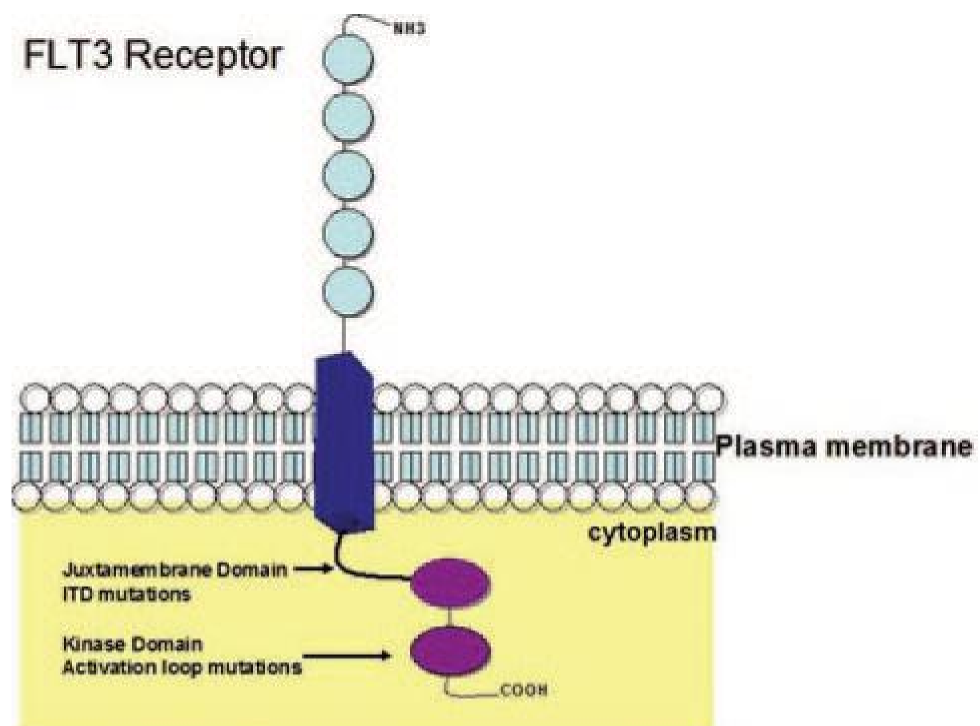


Figure 13. Flt-3 receptor (Parcells, et al., 2006)

2.8.2.2. Signaling through Flt3 receptor

The binding of Flt3 ligand (FL) to Flt3 receptor triggers the PI3K (Phosphatidylinositol 3-Kinase) and Ras pathways, leading to increased cell proliferation and the inhibition of apoptosis, (Lavagna-Sevenier, et al., 1998), (Figure 14). PI3K activity is regulated through various interactions between Flt3, SHCs (SH2-containing sequence proteins) and other proteins, such as SHIP (SH2-domain-containing Inositol Phosphatase), SHP2 (SH2-domain-containing protein tyrosine Phosphatase-2), Cbl (a proto-oncogene) and GAB2 (GRB2 Binding protein). Activated PI3K stimulates downstream proteins such as PDK1 (3-Phosphoinositide-dependent Protein Kinase-1), Akt1/PKB (Protein Kinase-B) and the mTOR (mammalian Target of Rapamycin), which initiate the transcription and translation of crucial regulatory genes through the activation of S6K (p70 S6 kinase) and the inhibition of 4E-BP1 (eukaryotic initiation factor 4E-Binding Protein) (Zhang and Broxmeyer, 1999). In addition, PI3K activation blocks apoptosis through phosphorylation of the pro-apoptotic BCL2 (B-Cell CLL/Lymphoma-2)-family protein BAD (BCL2 Associated Death Promoter). Activated Flt3 also associates with GRB2 (Growth Factor Receptor-Bound Protein-2) through SHC that activates Ras and stimulates downstream effectors such as Raf, MEK (MAPK/ERK Kinases), p38, ERK1/2 (Extracellular-signal Regulated Kinase), and the 90-kDa RSK (Ribosomal protein S6 Kinase). These downstream effectors activate CREB (cAMP Response Element Binding protein), Elk and STAT (Signal Transducer and Activators of Transcription), which lead to the transcription of Flt-3 mRNA and translation to Flt-3 protein involved in proliferation.

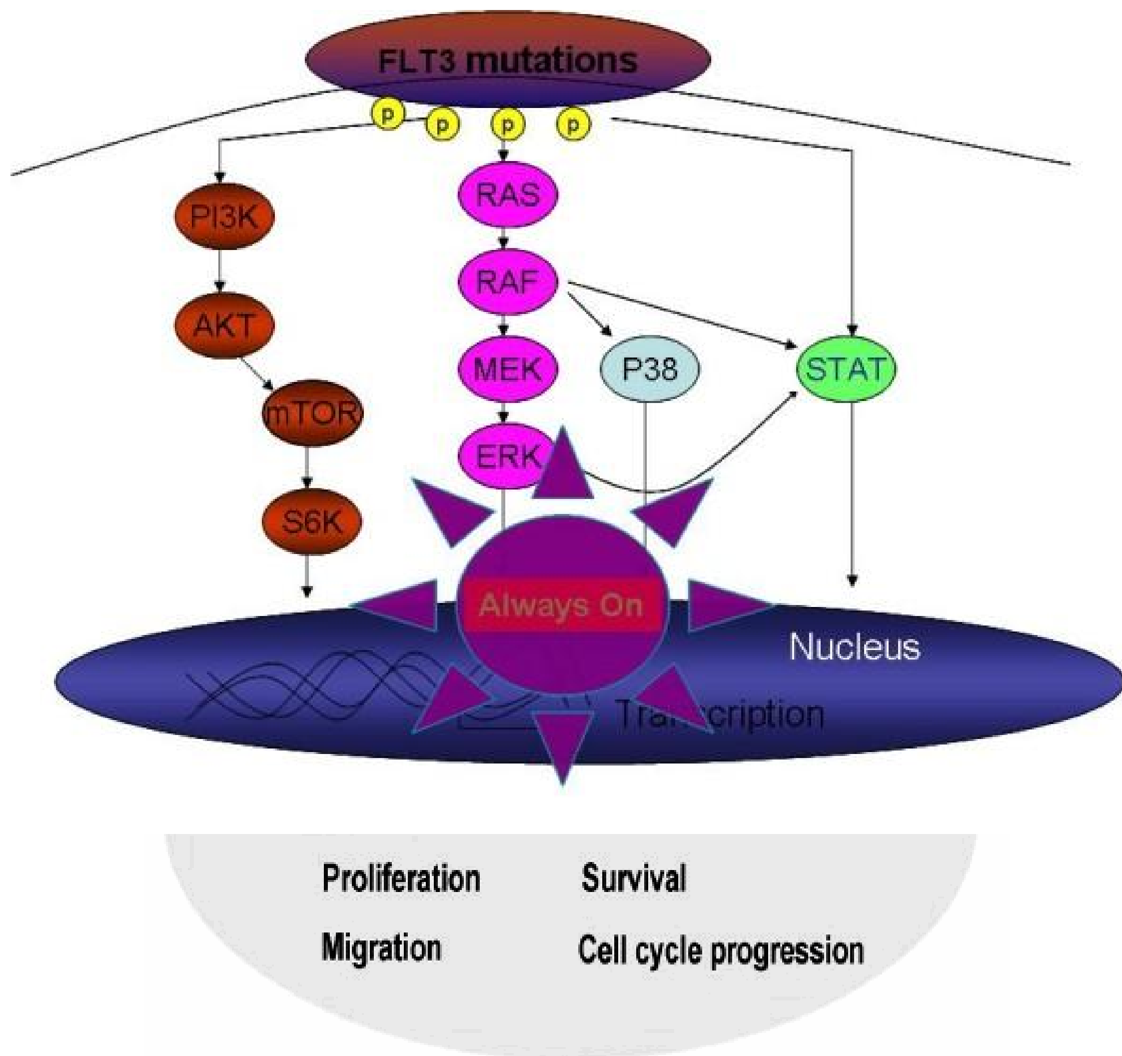


Figure 14. Signal transduction through Flt3 receptor

2.8.2.3. Flt-3 and acute myeloid leukemia (AML)

Self renewal and growth factor- independent proliferation are two important trials for oncogenesis. Clinical and experimental evidence both indicate that *FLT-3* is a proto-oncogene with the capacity to enhance survival and proliferation of leukemic blast cells (Parcells, et al., 2006).

FLT-3 gene is mutated in about 30% of AML patients and in a subset of ALL patients (Cools, et al., 2004). Two types of *FLT-3* gene mutations have been attributed to the constitutive activation of the Flt-3 receptor: internal tandem duplication mutation of 3-

400 bp that map the juxtamembrane domain of the receptor and contributes to 17-26% of AML patients, and single amino acid mutations (Parcells, et al., 2006; Small, 2006) most notably the substitution of tyrosine, histidine or valine for aspartic acid at position 835 within the activation loop of the kinase domain of the receptor (Parcells, et al., 2006).

2.8.2.4. Asp835tyr point mutation

This is a missense point mutation in the kinase domain that confers constitutive activation of the Flt-3 receptor. It is observed in 7% of AML cases and is easily identified because it results in the loss of an EcoRV restriction site. The normal aspartate residue is integral for regulating the activation loop, and evidenced by its conservation across several receptor tyrosine kinase subfamilies (Parcells, et al., 2006; Scholl, et al., 2005). The substitution stabilizes the "open" ATP- binding conformation of the activation loop (Till, et al., 2001).

The simultaneous presence of both types of mutations, so- called Flt-3 dual mutations, has been reported in 1-3% of AML cases (Chem, et al., 2005).

2.8.2.5. Flt-3 Inhibitors

Fms- like tyrosine kinase receptor 3 is an appropriate candidate for targeted therapy because it is expressed in many hematopoietic malignancies, it is the most frequent molecular abnormality in AML, it confers a poor prognosis, and its signaling cascade has been implicated in multiple tumorigenic pathways. Currently, there are several small- molecule therapies at various stages of development that target mutant forms of Flt-3 receptor (Parcells, et al., 2006). These inhibitors include: midostaurin (PKC412), sorafinib (BAY- 93006), and sunitinib (SU11248) (Small, 2008). All of the Flt-3

inhibitors being studied are heterocyclic compounds with a purine ring- like subunit that competitively inhibits ATP- binding to Flt-3 (Levis and Small, 2004).

However, acquired resistance to small- molecule inhibition has been identified in AML blasts harboring constitutively active Flt-3 receptors (Clark, et al., 2004). Because of genetic plasticity of cancer, leukemic cells can accumulate additional mutations that render Flt-3 signaling dispensable. Also, Flt-3 mutations can occur at a stage later than the leukemic stem cell, where an Flt-3 inhibitor would not affect the leukemic stem cell, which must happen to achieve a cure. So, the best approach was to combine these agents with conventional chemotherapy (Small, 2008).

2.9. Aims of the study

This study aims to select ssDNA aptamers from a synthetic library of 90 bases long ssDNA sequences, each having a 45 nucleotide long random region, flanked by two fixed regions. The selection process was performed against the active tyrosine kinase domain of both, the normal and mutant forms of Flt3 enzyme by the SELEX method. The selected aptamer was investigated for its relationship and homology with other anti-cancer/ anti-proliferative aptamers, which has a promisimg therapeutic potential in AML patients.

3. Materials and methods

3.1 Instruments and equipment

Thermal cycler (MJ Research, USA)

Gel documentation system (UVP, USA)

Spectrophotometer (Smart Spec TM plus, BIORAD, USA)

Electrophoresis chambers (Clever Scientific, UK)

Electrophoresis power supply (Power Pac Basic TM, BIORAD, USA).

Ultra-viol transilluminator (UVP, USA)

Autoclave (Selecta, Spain)

Incubator (flii Galli, Italy)

Centrifuge (Biofuge, Heraeus, Germany)

Digital balance (Denver, UK)

Vortex mixer (HARRIS, UK)

ELISA reader (Tipo, UK)

Pipettes (Witopet, Germany)

PCR tubes (Bio basic inc, Canada).

3.2.Reagents

Flt-3 active enzyme (millipore, USA),

Flt-3 (D835Y) active enzyme (millipore, USA),

Tyrosine kinase activity assay kit (Chemicon, USA),

µMacs GST isolation kit (MiltenyiBiotec, Germany),

µMacs microcolumns (MiltenyiBiotec, Germany),

µMacs separator (MiltenyiBiotec, Germany),

ssDNA library (Medland certified company, USA),

Synthesis of aptamers (Medland certified company, USA),

Primers (Medland certified company, USA),

Plasmid sequencing primer (Fermentas, France),

Taq DNA polymerase (Promega, USA),

PCR Green Buffer (Promega, USA),

Deoxy nucleotides triphosphate (dNTPs) (Promega, USA),

Nuclease free water (Promega, USA),

DNA ladder (Promega, USA),

PGEM-T and PGEM-T easy vector cloning system (Promega, USA),

E-coli starin MJ107 (Fermantas, France),

LE-agarose powder (Promega, USA),

Low melting point agarose (Promega, USA),

TE-saturated phenol (Promega, USA),

Pure Yield™ Plasmid miniprep System (Promega, USA)

DNA plasmid transformation kit (Fermantas, France),

Ligase enzyme (Fermantas, France),

Ligase buffer 2X (Fermantas, France),

Binding buffer (Promega, USA),

Elution buffer (Promega, USA),

Tris-Borate-EDTA 10X (Bio Basic Inc, Canada),

TE buffer (Promega, USA),

Loading dye (Promega, USA),

Ethidium bromide (Promega, USA),

Absolute ethanol (Reidel-de Haen, Germany),

NaCl (Promega, USA),

Sodium acetate (Reidel-de Haen, Germany),

HEPES (Promega, USA),

LB media (agar) (Invitrogen. USA),

LB media (broth) (Invitrogen. USA),

Ampicillin powder, (Promega, USA),

5-Bromo-4-chloro-3-indolyl-β-D galactoside (Xgal) (Bio Basic Inc, Canada),

Isopropyl-1-thio-β-D-Galactoside (IPTG) (Bio Basic Inc, Canada),

$MgCl_2$ (Promega, USA),

EDTA (Invitrogen. USA),

Di-Methyl Sulphoxide (DMSO) (Gibco, USA),

QIAquick PCR purification kit (QIAgen, USA)

3.3. Methods

3.3.1. ssDNA library and primer design

The initial template was obtained via chemical synthesis using cyanoethyl phosphoroamitide chemistry by the Medland Company, USA.

DNA fragments were 90 bases in length**,** with 45 nucleotides random region flanked with defined sequences of the forward and reverse primers, and restriction sites for BamHI, HindIII and EcoRI restriction enzymes.

5`-GGGATGGATCCAAGCTTACTGG**45N**GGGAAGCTTCGATAGGAATTCGG-**3'**

Where N is one of the four deoxynucleotides; A, T, C or G, so, this library will give theoretically 4^{45} different sequences, which is equal to $1.24x10^{27}$ sequences.

The sequences of the forward and reverse primers were as follows:

Forward primer: 5' GGGATGGATCCAAGCTTACTGG 3'

Reverse primer: 5' CCGAATTCCTATCGAAGCTTCCC 3'

3.3.2. Small scale amplification

In order to eliminate damaged templates and provide multiple copies of the original ones, the chemically synthesized oligonucleotide pool is first amplified enzymatically by PCR before initiating a selection experiment. Optimal amplification conditions can be determined before large-scale amplification to prepare the initial pool of dsDNA by carrying out such small-scale trials.

The optimal conditions were as follows:

One hundred nanograms of the initial template were used in the small scale amplification step which represents about $1x10^{12}$ different sequences. This was calculated according to the formula: number of copies = (amount (ng) * $6.022x10^{23}$) / (length * $1x10^{9}$ * 650), where $6.022x10^{23}$ is Avogadro's number, and 650 is the average

weight of a base pair (bp) in Daltons, (Andrew Staroscik, 2004). The total volume of PCR reaction was 50 µl of PCR mix containing green buffer, 200 µM of each dNTPs mix (Promega, USA), 1 µM of the forward primer 5' GGGATGGATCCAAGCTTACTGG 3' and 1 µM of the reverse primer 5' CCGAATTCCTATCGAAGCTTCCC 3' (Medland, USA), and 2.5U of Taq DNA polymerase (Promega, USA). PCR program was as follows: heating up to 95˚C for 5 minutes as an initial denaturation step, then thermally cycling seven times of 15 seconds at 95˚C, 15 seconds at 50˚C as annealing temperature, 20 seconds at 72˚C as an extension step. These seven cycles were then followed by the final extension step at 72˚C for two minutes.

3.3.3. Large scale amplification

Ten micrograms of the chemically synthesized template were amplified to prepare the initial pool of the double stranded DNA (dsDNA), which represents approximately $1x10^{14}$ different sequences, in 5000 µl of total volume PCR reaction mix divided into 100 PCR tubes, each containing 50 µl of PCR reaction mix with same conditions optimized in small-scale PCR as described above.

3.3.4. Small scale asymmetric PCR

In asymmetric PCR one primer is added in vast excess over the other. This method is used to produce ssDNA from dsDNA.

The small-scale amplification by asymmetric PCR was used to optimize the preparation of initial pool of ssDNA. It was performed by amplifying 5 µl of dsDNA PCR product obtained from the large-scale amplification step in 50 µl of total volume PCR reaction in PCR mix containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20mM Tris HCl (pH 8.75), 0.1% Triton-x 100, 0.1 mg/ml BSA, 1 µM of the forward primer, 0.02µM of the reverse primer, 200 µM of each dNTPs mix (Promega, USA), 1.5 U of Taq DNA polymerase (Promega,USA).

Asymmetric PCR program was as follows: 5 minutes at 95°C, then 25 cycles of 15 seconds at 94°C, 15 seconds at 55°C, 20 seconds at 72°C, followed by final extension step of 2 minutes at 72°C.

3.3.5 Large scale asymmetric PCR

Twenty percent of the double stranded oligonucleotide pool were amplified by asymmetric PCR in 5000 µl of total volume PCR reaction mix divided into 100 PCR tubes each containing 50 µl total volume in the same PCR conditions described in small-scale asymmetric PCR.

3.3.6. Agarose gel electrophoresis

PCR products from all amplification reactions were detected by electrophoresis on 4% agarose gel.

3.3.6.1. Preparation of 4% agarose gel

An adequate volume of electrophoresis 1X TBE buffer was prepared by diluting one volume of 10X TBE buffer (Promega, USA) in nine volumes of distilled water. Then 2 grams of agarose powder were added (Promega,USA) into 50 mL of 1X TBE buffer and the mix was melted in a microwave with swirling to ensure even mixing and clear appearance. Then, 10 µl of 2.5mg/ml ethidium bromide were added to the mix and poured into the mini gel casting platform and allowed to harden.

3.3.6.2. Loading the samples

The gel casting platform containing the set gel was placed in the electrophoresis tank soaked in sufficient volume of 1xTBE buffer. About 10 µl were loaded into the well. Each run has 3 µl of DNA ladder mixed with 3 µl of loading dye to determine the size of the bands of the PCR product. To begin the electrophoresis, the leads were connected with the power supply and the gel was run at 100 volt for 20 minutes. The DNA was visualized by placing on a UV light source where it was photographed directly.

3.3.6.3 Preparation of 3% low melting point agarose gel

One and half grams of low melting point agarose (Promega, USA) were melted in 50 ml 1xTBE buffer by following the same steps for the 4% agarose gel preparation.

3.3.7. Extraction of ssDNA fragments from low melting point agarose gel

After the asymmetric PCR reaction was run, the ssDNA yield must be extracted efficiently in order to produce pure single stranded oligonucleotide library.

The samples were loaded into 3% low melting point agarose gel and electrophoresed. The method of extracting the ssDNA yield was as follows:

The desired bands of ssDNA were cut using sterile scalpel and placed in 1.5 ml eppendorf tubes. Then, 300 μl of 1xTBE buffer pH 8.0 (Promega, USA) were added and the tubes were heated at 65°C for 30-45 minutes until the gel has dissolved. An equal volume of TE saturated phenol (pH 8.2) was added to the tubes and vortexed for about 1 minute to produce a milky appearance solution; which was centrifuged for 15 minutes at 13,000 rpm. After centrifugation, the supernatant was taken to a new eppendorf tube and 2.5 volumes of absolute ethanol and 0.1 volume of 3M sodium acetate were added, and the mixture was incubated at -20°C overnight to enhance the precipitation of ssDNA. On the next day, the mixture was centrifuged for 15 minutes at 13,000 rpm. Then, the supernatant was discarded and the small transparent pellet was washed with 700 μl of 70% ethanol to remove residual phenol and salts, followed by centrifugation for 10 minutes at 13,000 rpm. The pellet was then rehydrated with 20 μl of nuclease free water. This pool is ready to be used in the iterative cycles of the SELEX process.

3.3.8. In vitro selection process

3.3.8.1. Folding of ssDNA library

The ssDNA library was heated at 95°C for 10 minutes in 200 µl binding buffer which contains 100 mM HEPES (pH 7.5), 10 mM imidazole, 1 mM $MgCl_2$, and 150 mM NaCl (Promega, USA). Then, the mix was rapidly cooled into 4ºC for 5 minutes and then incubated on ice for 15 minutes to insure proper folding of ssDNA ligands into its 3D structures, after which, it was equilibrated at room temperature for about 10 minutes.

3.3.8.2. Negative selection

The folded ssDNA ligands were incubated with 50 microliters of µMacs anti-GST magnetic beads (MiltenyiBiotec, Germany) on ice for 30 minutes. Then a µMacs microcolumn was placed on a separator with high magnetic field and the mixture was let to flow through the microcolumn, and the flowthrough was collected. This step was the negative selection process performed in the first three rounds of selection in order to pick-up any sequence that would stick to the beads before being bound to the target of interest to eliminate false background selection of high affinity ligands to GST.

3.3.8.3. Incubation of active enzymes with µMacs anti-GST magnetic beads

After the negative selection was performed, the flowthrough which contained the ssDNA library was incubated with the active tyrosine kinase domain of both Flt-3 enzymes, the wild type and the mutant form D835Y (1µg of each enzyme for the first round and 0.5 µg for the next rounds) for 1 hr at 37°C. After that, 50 µl of µMacs anti-GST magnetic beads were added and incubated on ice for 30 minutes.

Note: normal and mutant forms of Flt-3 enzyme were run at similar concentrations in the same reaction tube up to the fifth round of selection, in order to elicit, if present, similar domains that the aptamer will bind to.

A microcolumn is placed on the magnet separator, and the mixture was let to flow, then the microcolumn was washed three times for the first five rounds and six times for the final three rounds with 200 µl binding buffer to remove any non specific binders and to enhance efficient partition.

3.3.8.4. Elution of ssDNA

The ssDNA bounded to the enzymes and the magnetic beads would be eluted from the microcolumn matrix, by removing the column from the magnet separator stand and adding 50 µl of binding buffer.

The collected eluate contained the ssDNA that binds the enzyme which is bound to the magnetic beads.

3.3.8.5. Amplification of ssDNA eluate

Because of the high complexity of the initial oligonucleotide library it is normally expected to get only few functional oligonucleotides in result of the selection step.

ssDNA was amplified by PCR using the same conditions described above except for the duration of initial denaturation which was 10 minutes instead of 5, and the number of cycles which was 15 cycles instead of 7, and then performing asymmetric PCR in the same conditions described above to produce ssDNA candidate mixture for the next round of selection.

3.3.8.6. Negative control PCR

After each round, a negative control of PCR was performed to ensure that there is no contamination that interferes with the enrichment process of aptamers which gives false selected sequences later in cloning and sequencing.

3.3.8.7. Rounds of selection

Eight rounds of SELEX were performed to select anti-Flt-3/ anti-Flt-3(D835Y) aptamers with conditions summarized in Table (2).

Table (2). Conditions of each round of SELEX

Round	Number of PCR cycles	Quantity of ssDNA (µg)	Quantity of normal and mutant enzyme at the same tube (µg)	Quantity of normal enzyme (Flt-3) (µg)	Quantity of mutant enzyme (Flt-3-D835Y) (µg)	Incubation temperature	Number of washings	Incubation time
1	15	-	2	1	1	37° C	3	One and half hour
2	15	1	1	0.5	0.5	37° C	5	One and half hour
3	15	1	1	0.5	0.5	37° C	5	One and half hour
4	15	1	1	0.5	0.5	37° C	5	One and half hour
5	15	1	1	0.5	0.5	37° C	5	One and half hour
6	15	1 (0.5 for each enzyme)	-	0.5	0.5	37° C	6	One and half hour
7	15	1 (0.5 for each enzyme)	-	0.5	0.5	37° C	6	One and half hour
8	15	1 (0.5 for each enzyme)	-	0.5	0.5	37° C	6	One and half hour

3.3.9. Cloning

By iteratively executing the procedures of selection, amplification and conditioning during the SELEX process, the complexity of the original library is reduced and target-binding candidates are enriched. Therefore, the final pool of the eighth selection round was cloned into the bacterial vector PGEM-T to determine the sequence of each individual aptamer, as follows:

3.3.9.1. X-Gal preparation

Preparation of 3% X-Gal was performed by dissolving 0.1gram of X-Gal powder (Bio Basic Inc, Canada) in 3 mL of DMSO (Gibco, USA) to a final concentration of 33 mg/ml.

3.3.9.2. IPTG preparation

Preparation of IPTG was performed by dissolving 1gram of IPTG powder (Bio Basic Inc, Canada) in 50 ml of autoclaved distilled water to a final concentration of 10 mM.

3.3.9.3. LB solid media preparation

Preparation of LB solid media was performed by dissolving 16 grams of LB powder in 500 ml of distilled water followed by heating the media until completely dissolved the powder. Then, the media was autoclaved for 15 min at 121°C and after cooling the media to 50°C about 25 mg of ampicillin powder were dissolved in the media to a final concentration of 50µg/ml. Then, 5 ml of 10 mM IPTG were added to a final concentration of 0.1 mM, and about 0.3 ml of X-Gal were added to the media to a final concentration of 20µg/ml. Then, after well mixing, the media was poured into Petri dishes and stored at 4°C.

3.3.9.4. LB broth media preparation.

Preparation of LB broth media was performed by dissolving 2 grams of LB broth powder in 100 ml of distilled water followed by heating the media until complete dissolving of powder. The media was then autoclaved for 15 min at 121°C. After cooling the media to 50°C, 5 mg of ampicillin powder was added to a final concentration of 50µg/ml and then poured into sterile 1.5 ml microcentrifuge tubes (1ml/tube) and stored at -20°C.

3.3.9.5. Preparation of *E.coli* competent cells

Under aseptic conditions, 0.5 ml of liquid LB media without ampicillin was added to powder of freeze-dried *E.coli* and mixed well. Inoculating loop was used for streaking the mixture on nutrient agar plate which is then incubated overnight at 37°C. Then, one colony of bacterial culture was moved from overnight nutrient plate using inoculating loop into 1 ml of transformamide C-Medium (Fermantas, France). The culture was suspended by gently mixing and incubation of the tube in a shaker at 37°C for 2 hours.

3.3.9.6. Purification of PCR product

After the eighth round of selection, the obtained aptamer should be purified from other components of the final dsDNA product. The purification was performed following the QIAquick PCR purification kit (QIAgen, USA) as follows:

Five volumes of the PB buffer (provided by the kit) were added to 60 µl of PCR product and mixed, and then the sample was applied to the QIAquick column and placed in a provided 2 ml collection tube and then centrifuged for 30 seconds at 13,000 rpm. The flow-through was discarded and the columns were washed with 750 µl of PB buffer and then centrifuged for 1 minute at 13,000 rpm. Again the flow-through was discarded and

the columns were centrifuged for another minute. In order to elute the purified DNA, 50 µl of EB buffer were added to the QIAquick columns placed on clean 1.5 microcentrifuge tubes and centrifuged for 1 minute at 13,000 rpm. After that, the eluted DNA was collected and stored at -20°C.

3.3.9.7. Ligation

Ligation of the purified dsDNA to the PGEM-T vector was performed following the protocol of the PEGM-T and PEGM-T easy vector system kits (Promega, USA), by mixing 3 µl of the PCR product with 1 µl of T4 DNA ligase enzyme and 1 µl of PEGM-T and PEGM-T easy vector plus 5 µl of 2x Rapid Ligation buffer. The mixture was incubated for one hour at room temperature.

3.3.9.8. Transformation and growth

After preparation of E-coli competent cells from a fresh culture plate in C-Medium as described above, the tube was spun at 13,000 rpm for 1 minute at room temperature. Then, the supernatant was discarded and the pelleted cells were resuspended in 300 µl of T-solution and incubated on ice for 5 minutes. The cells were spundown again for 1 minute at 13,000 rpm at room temperature and then the supernatant was removed and the cells were resuspended in 120 µl of T-solution and then incubated on ice for 5 minutes. During that, LB-ampicillin agar plates are pre-warmed at 37°C for 20 minutes.

Preparations of DNA for transformation was performed by taking 5 µl of ligation mixture into new microcetrifuge tube and placing it on ice for 2 minutes. Then, 50 µl of the resuspended cells were added to the tube that contains DNA and incubated on ice for 5 minutes. Then, the cells were plated on pre-warmed LB-ampicillin agar plates and incubated overnight at 37°C. After growing the transformed cells, white and blue

colonies were screened to determine successful cloning which is indicated by the presence of white colonies.

3.3.9.9. Preparation of plasmid for sequencing

Plasmid extraction was performed by the Pure Yield™ Plasmid miniprep System (Promega, USA) by incubation of each white colony in 1 ml of LB-ampicillin broth media overnight at 37°C on shaking platform. Six hundred microliters of the bacterial cells grown in LB broth medium were lysed by the addition of 100 µl of cell lysis buffer, and the tubes were mixed by inversion. Then, 350 µl of neutralization solution were added and the tubes were centrifuged for 3 minutes at 13,000 rpm. After that, the supernatant of each tube was transferred to a PureYield™ minicolumn and placed into a PureYield™ collection tube, and then the collection tubes were centrifuged for 15 seconds at maximum speed. After that, the flowthrough was discarded and 200 µl of Endotoxin Removal Wash were added and centrifuged at maximum speed for 15 seconds. This step was proceeded by the addition of 400 µl of Column wash solution to the microcolumns and centrifuged again at 13,000 rpm for 30 seconds. The microcolumns were transferred to clean 1.5 ml microcentrifuge tubes and 30 µl of elution Buffer were added to each tube, let to stand at room temperature for 1 minute and then centrifuged for 15 seconds at maximum speed. The eluate containing the purified plasmid DNA was collected and stored at -20°C.

3.3.10. Sequencing

Extracted plasmids were sent for sequencing with the forward universal sequencing primer M13/pUC. The sequencing reactions were performed by Macrogen Inc., South Korea by the Applied Biosystems instrument.

3.3.11. Synthesis of aptamer sequences

Each aptamer sequence was obtained via chemical synthesis using cyanoethyl phosphoroamitide chemistry (Medland, USA) in 50 nanomole scale.

4. Results

4.1. Results of small and large scale dsDNA PCR amplification

The chemically synthesized oligonucleotides were amplified enzymatically by PCR before initiating a selection experiment in order to eliminate damaged templates and provide multiple copies of the original ones. Optimal amplification conditions were determined by small-scale PCR amplification prior to large-scale PCR amplification. The result of amplification was a 90 base pair long fragment as seen in Figure (15)

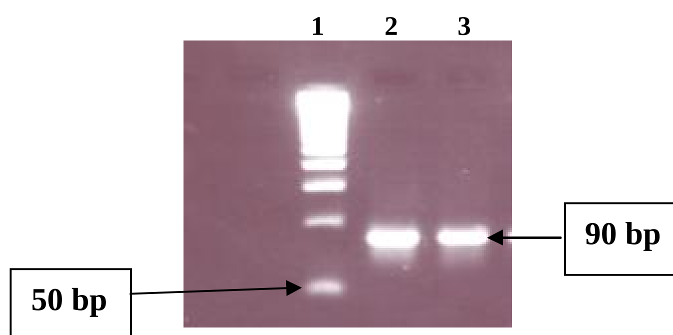


Figure 15. 4% agarose gel electrophoresis shows 90 bp PCR product of small-scale (lane 2) and large-scale (lane 3) amplification guided by 50bp ladder (lane 1).

4.2. Results of asymmetric PCR amplification

Optimal amplification conditions were determined by small-scale asymmetric PCR amplification prior to large-scale one. The result of asymmetric amplification showing the relevant 50 bp band is seen in Figure (16).

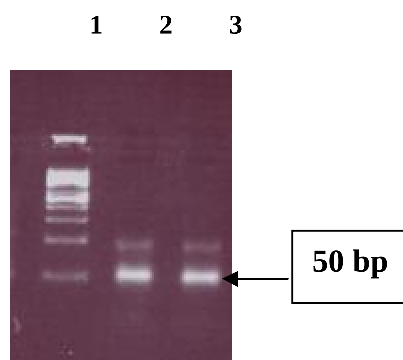


Figure 16. 3% LMP agarose gel electrophoresis shows 50 bp asymmetric PCR product of the small scale (lane 2) and large scale (lane 3) amplification, guided by 50 bp ladder (lane 1).

4.3. PCR products of eight rounds of selection

Eight rounds of SELEX were performed to select anti-Flt-3 aptamers. After round number five, the pool of ssDNA was separated into two parts, one for each enzyme, and SELEX rounds were run separately. About 10 μl of PCR product of each round were loaded into 4% of agarose gel and the results were as present in Figure (17).

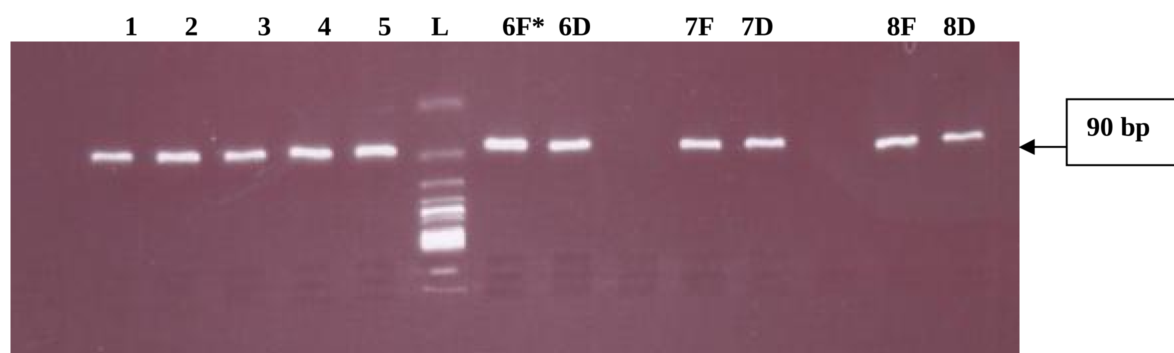


Figure 17. 4% agarose gel electrophoresis shows PCR product of each round of selections guided by 50 bp ladder (lane L).

*** F: nomal enzyme (Flt3), D: mutant form of the enzyme (D835Y)**

4.4. Colony pick up and Sequencing results

Successful colonies containing the plasmids of interest were picked up and the plasmids were purified using the Pure Yield™ Plasmid miniprep System (promega, USA). Twenty plasmids containing the anti-Flt3 aptamer and eighteen plasmids for the mutant one were sent and sequenced by Macrogen Incorporation, South Korea. Out of the 10^{14} different sequences in the starting library only one sequence of Anti-Flt-3/ D835Y aptamers was obtained:

5`-TCAAATGGTGTTGGTGGTTGGGGGATATGGGCGGTGGGAGGTACT-3'

The sequencing results were highly accurate and monitored by precise quality control system. Table (3) describes the sequence type of anti-Flt3 aptamer, its molecular weight, and the percentage of nitrogenous bases in the sequence obtained by the CLC main workbench program. It is obvious that the most frequent base with the highest percentage (43%) is guanine (G).

Table (3). Anti- Flt3 aptamer informative data.

Sequence type	ssDNA	
Weight	29.523 kDa	
Nucleotide	**Count**	**Frequency (%)**
Adenine (A)	18	20
Cytosine (C)	10	11
Guanine (G)	39	43
Thymine (T)	23	26

4.5. Primary and secondary Structures of selected aptamers

The secondary structures of anti-Flt-3 aptamer were predicted by computer modeling using the Mfold program (available at http://www.bioinfo.rpi.edu/applications/mfold/) described by Michael Zuker who developed an algorithm to predict nucleic acid folding and hybridization by free energy minimization using empirically derived

thermodynamic parameters. This program takes in consideration the factors that affect the folding of nucleic acid sequences, and the conditions of incubation, such as temperature of incubation and ionic strength.

The most stable secondary structure for Flt-3 aptamer having the smallest free energy is shown in Figure (18) with its primary and secondary structures.

There were another ten predicted secondary structures for the selected aptamer, and they are shown in Figures (19-28) arranged from the more stable structure with the lowest free energy to the least stable one.

5`GGGATGGATCCAAGCTTACTGG**TCAAATGGTGTTGGTGGTTGGGGGAT**

ATGGGCGGTGGGAGGTACTGGGAAGCTTCGATAGGAATTCGG-**3'**

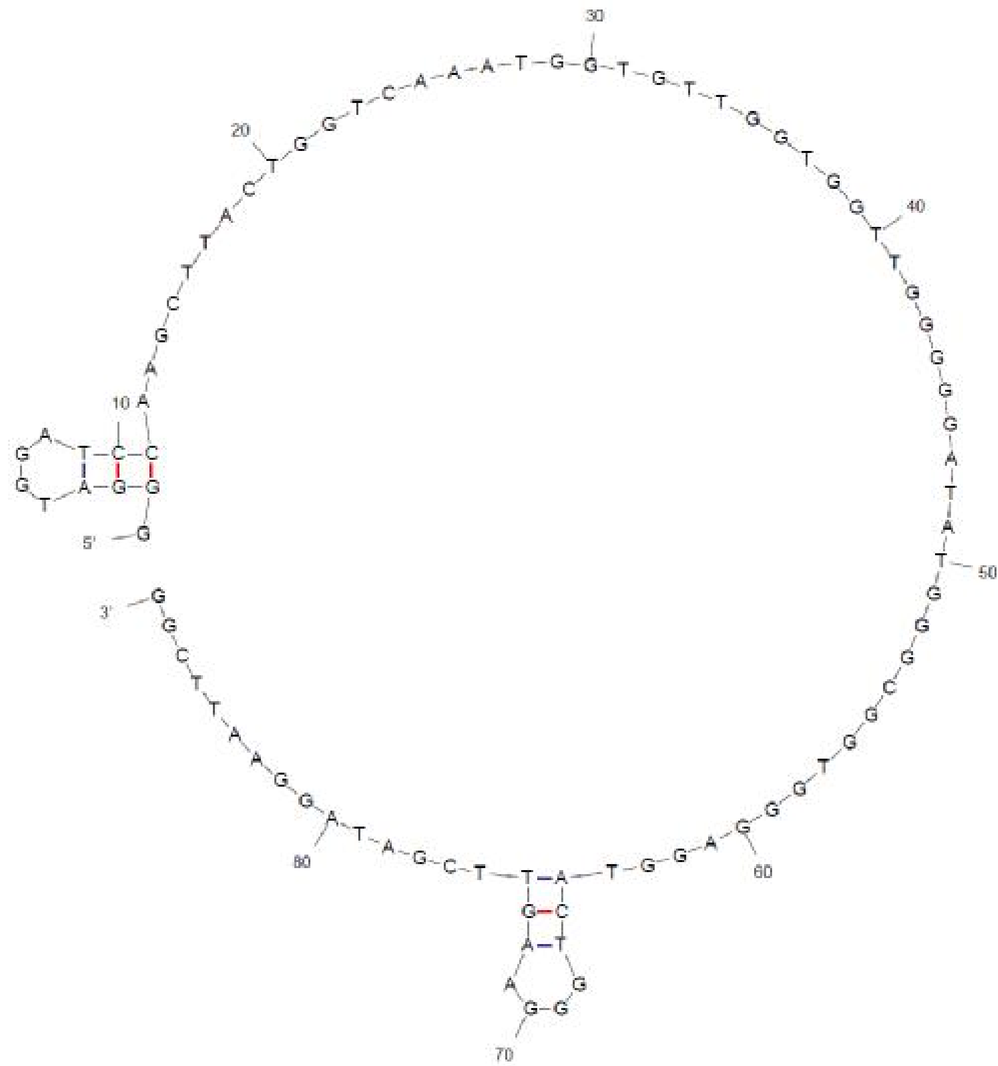


Figure 18. The primary structure and the predicted secondary structure of anti-Flt-3 aptamer with the lowest free energy (ΔG = - 2.71)

Figure 19. Predicted secondary structure of anti-Flt-3 aptamer (ΔG = - 2.61)



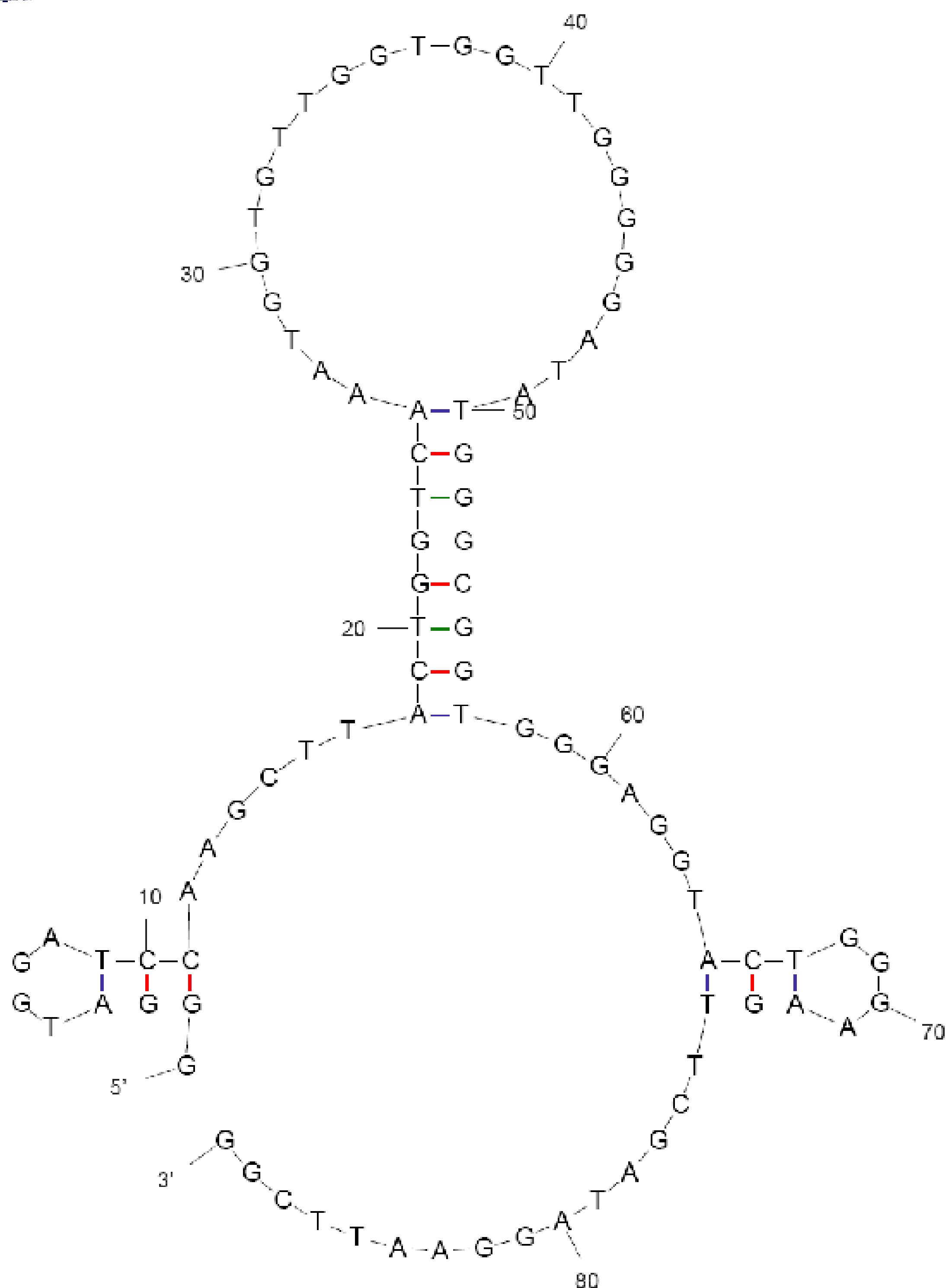


Figure 20. Predicted secondary structure of anti-Flt-3 aptamer (ΔG = - 2.52)

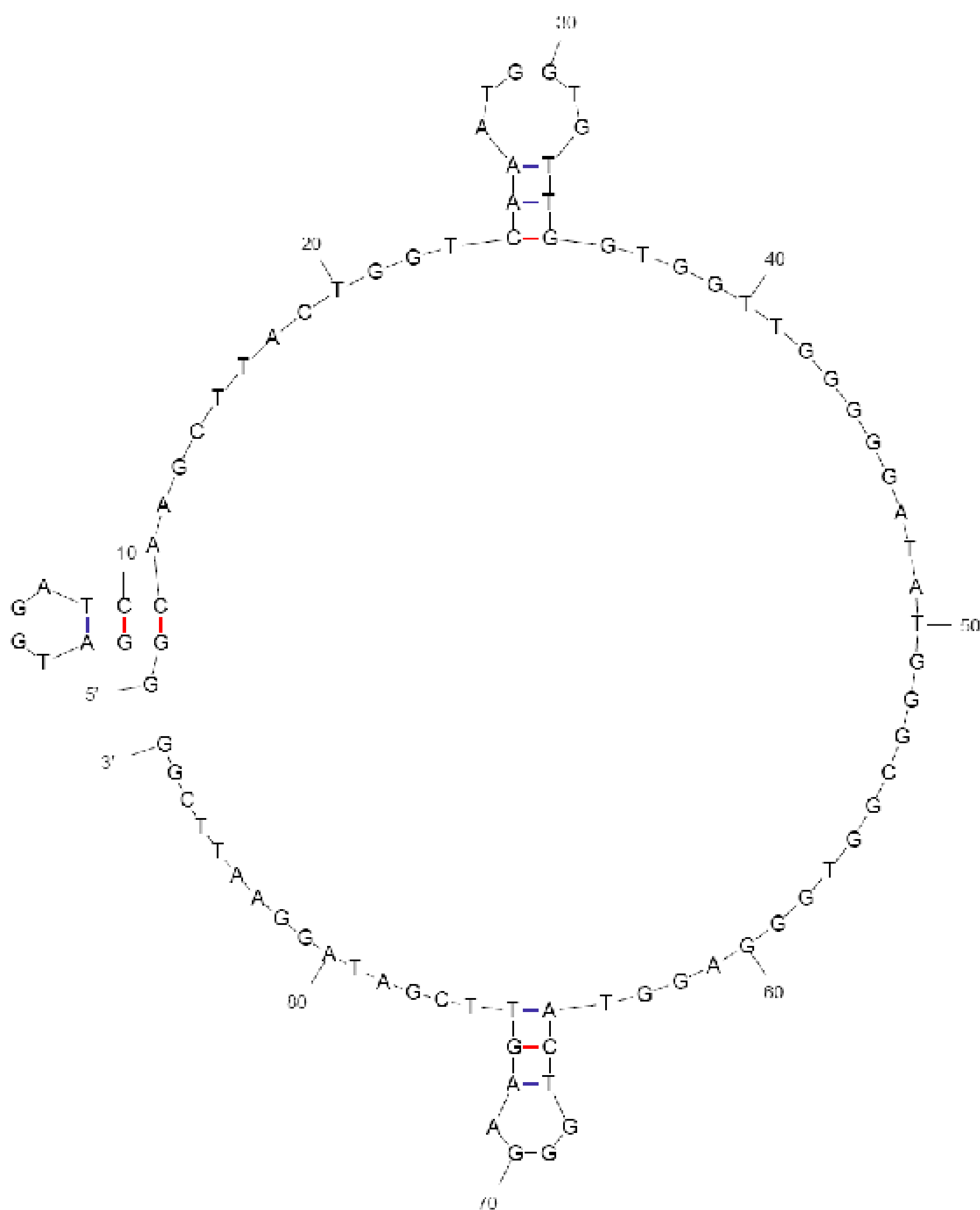


Figure 21. Predicted Secondary structure of anti-Flt-3 aptamer (ΔG = - 2.2)

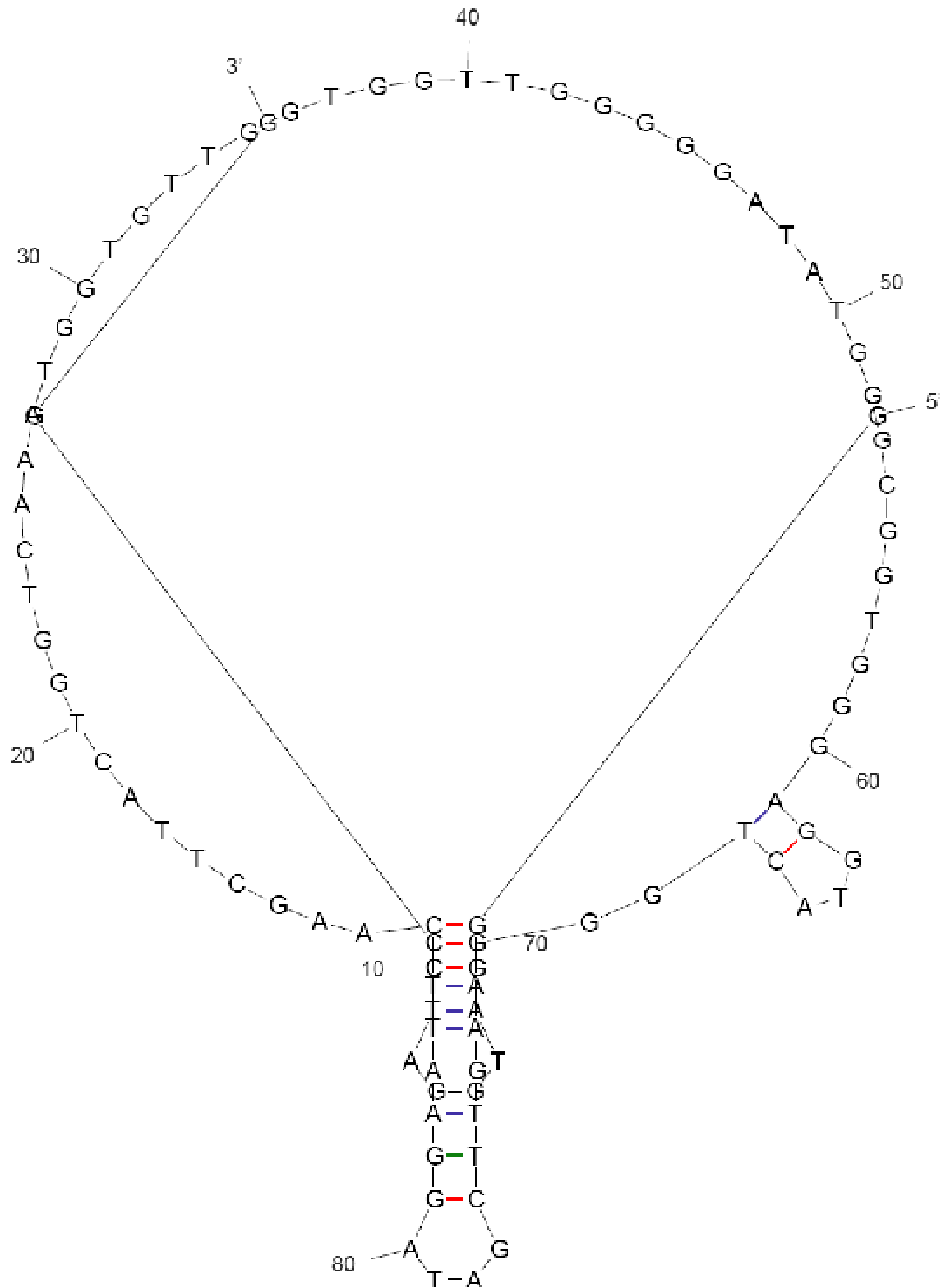


Figure 22. Predicted secondary structure of anti-Flt-3 aptamer (ΔG = - 2.1)

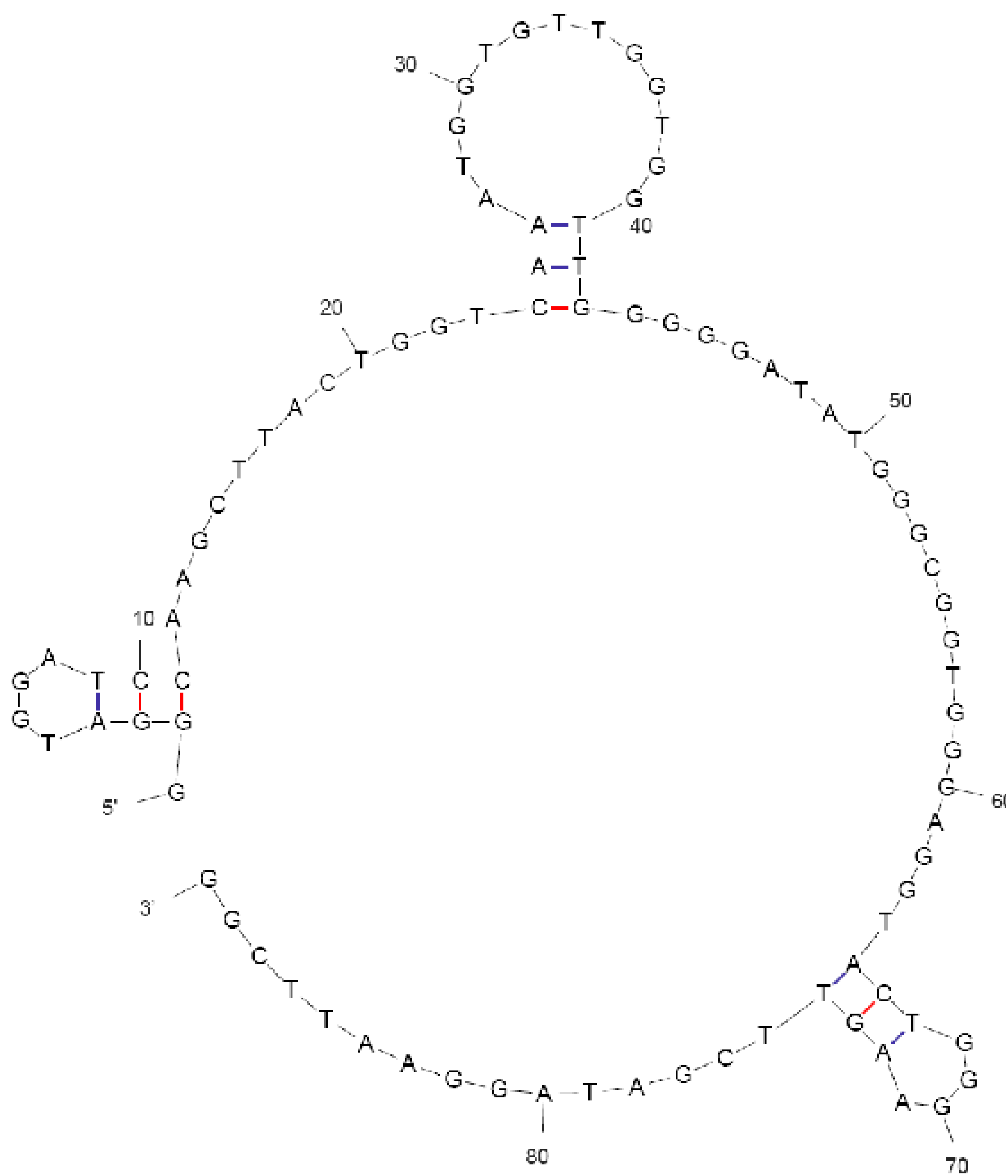


Figure 23. Predicted secondary structure of anti-Flt-3 aptamer (ΔG = - 1.6)

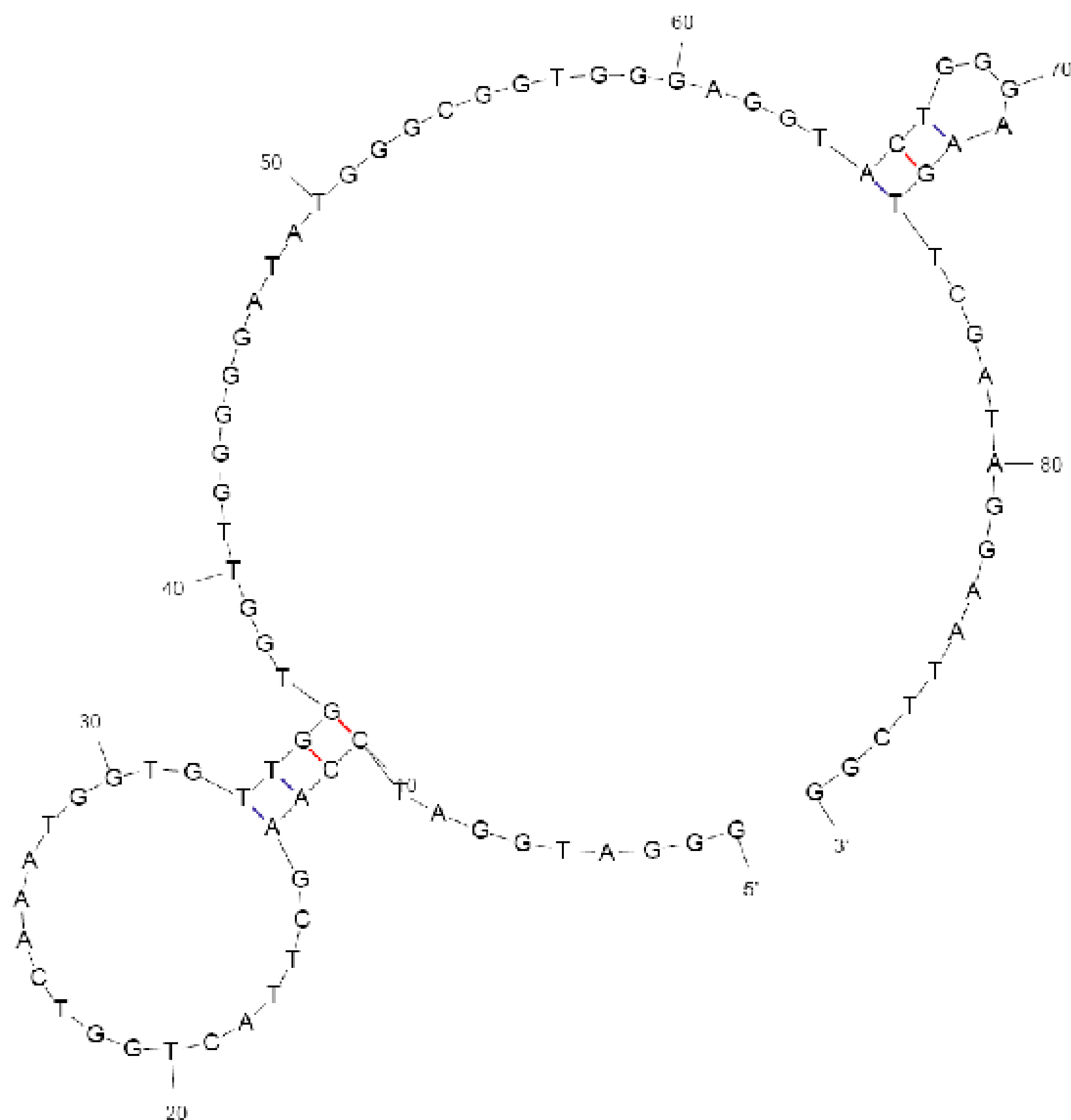


Figure 24. Predicted secondary structure of anti-Flt-3 aptamer (ΔG = - 1.37)

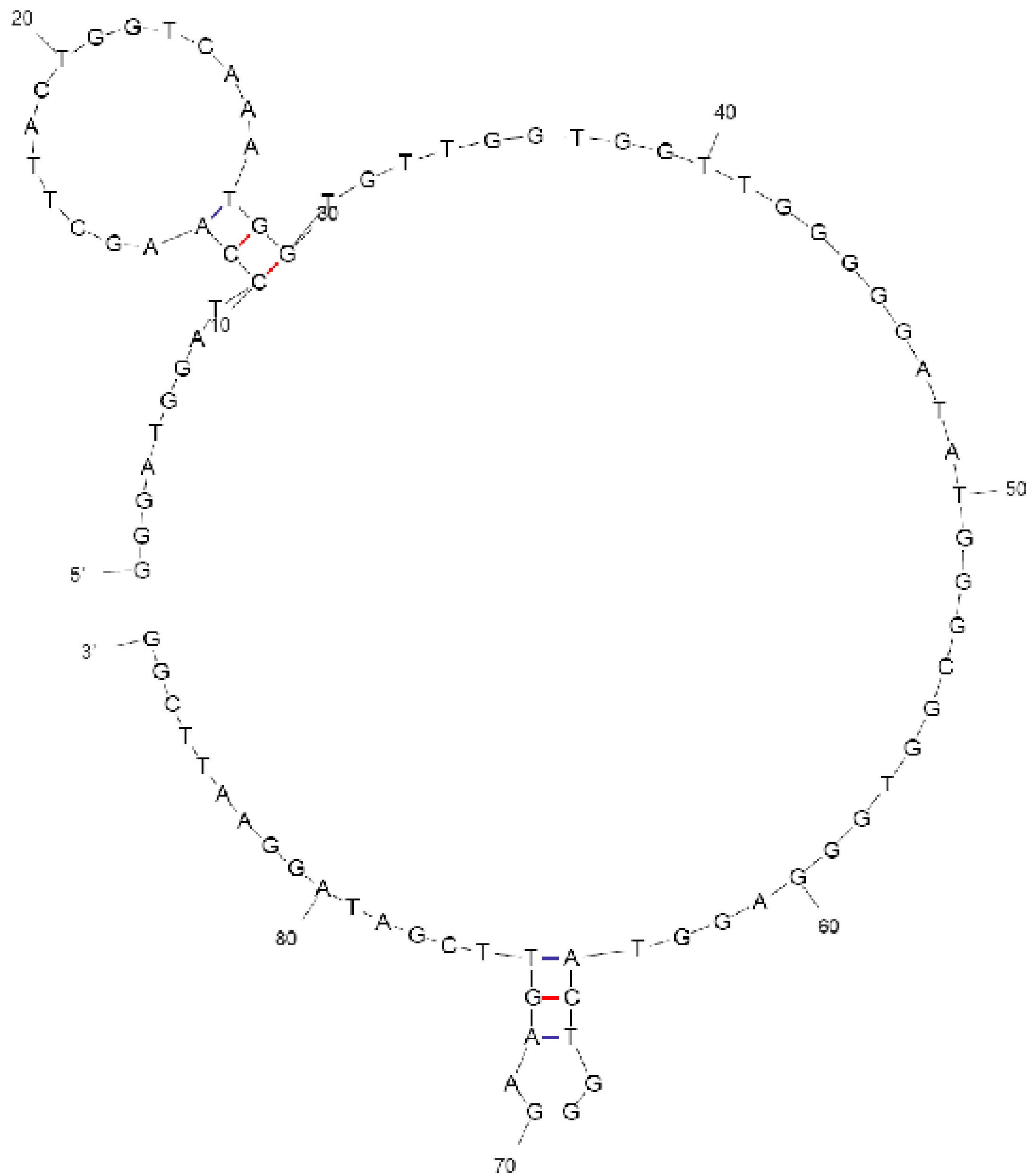


Figure 25. Predicted secondary structure of anti-Flt-3 aptamer (ΔG = - 1.13)

Figure 26. Predicted secondary structure of anti-Flt-3 aptamer (ΔG = - 0.79)

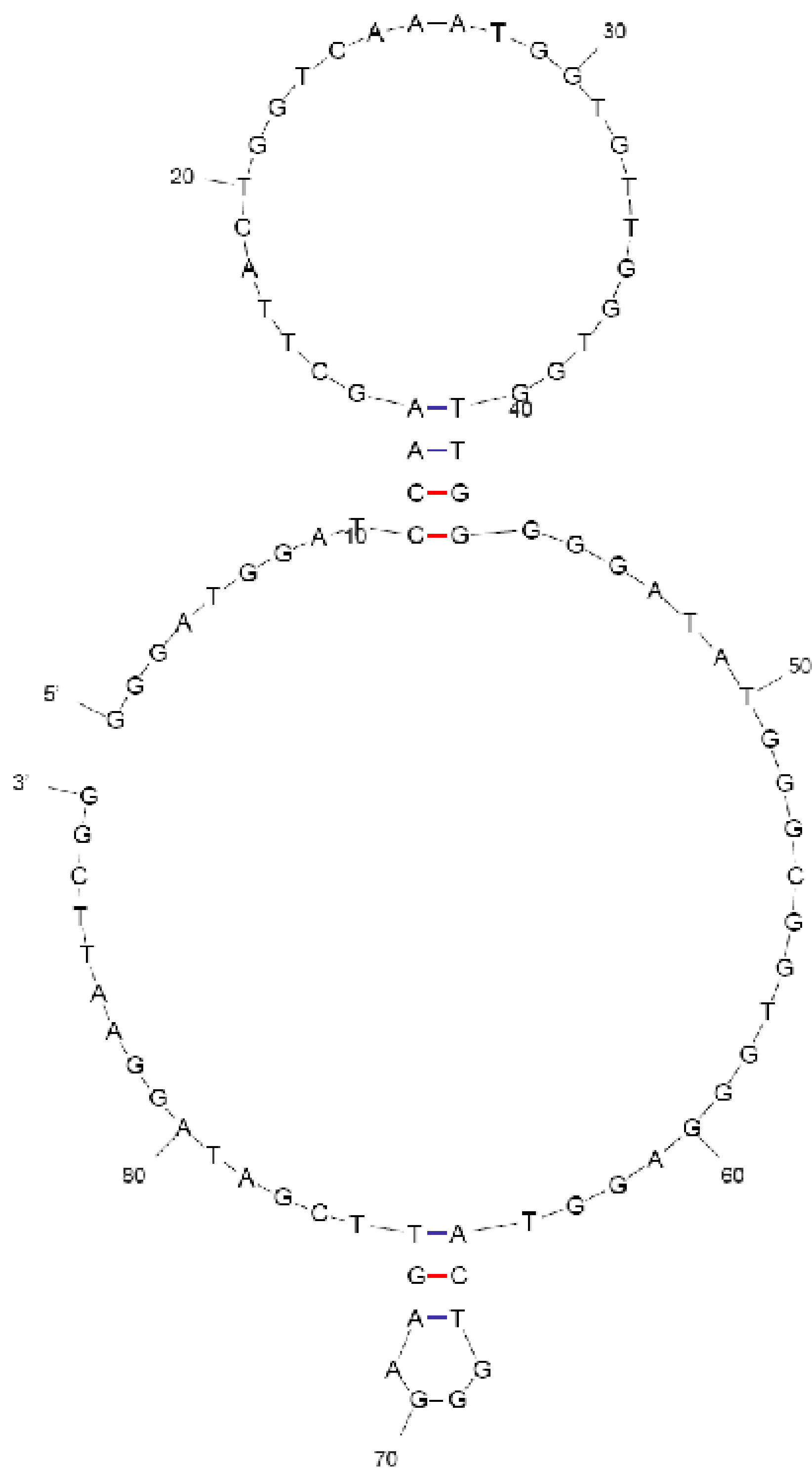


Figure 27. Predicted secondary structure of anti-Flt-3 aptamer (ΔG = - 0.66)

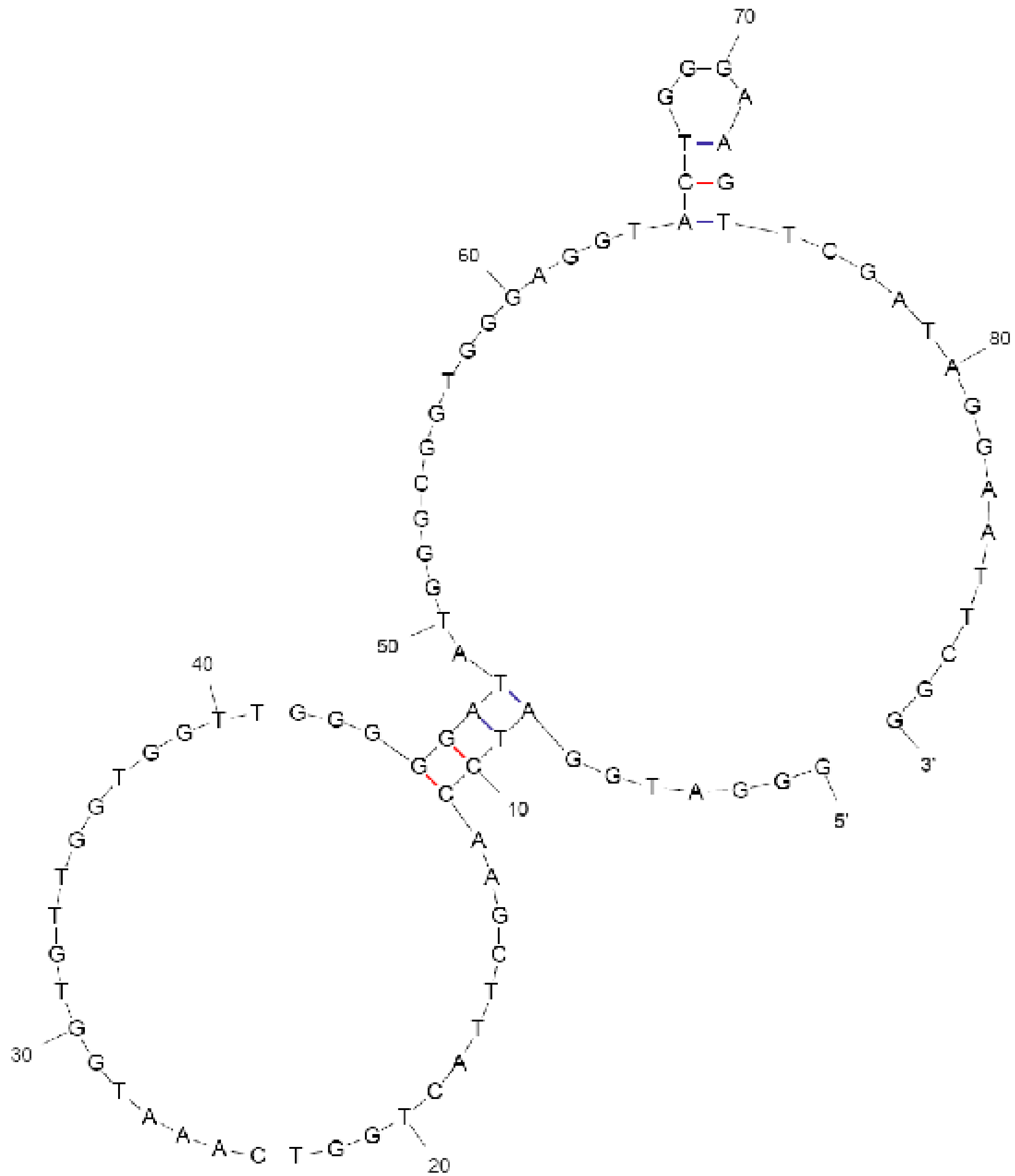


Figure 28. Predicted secondary structure of anti-Flt-3 aptamer (ΔG = - 0.42)

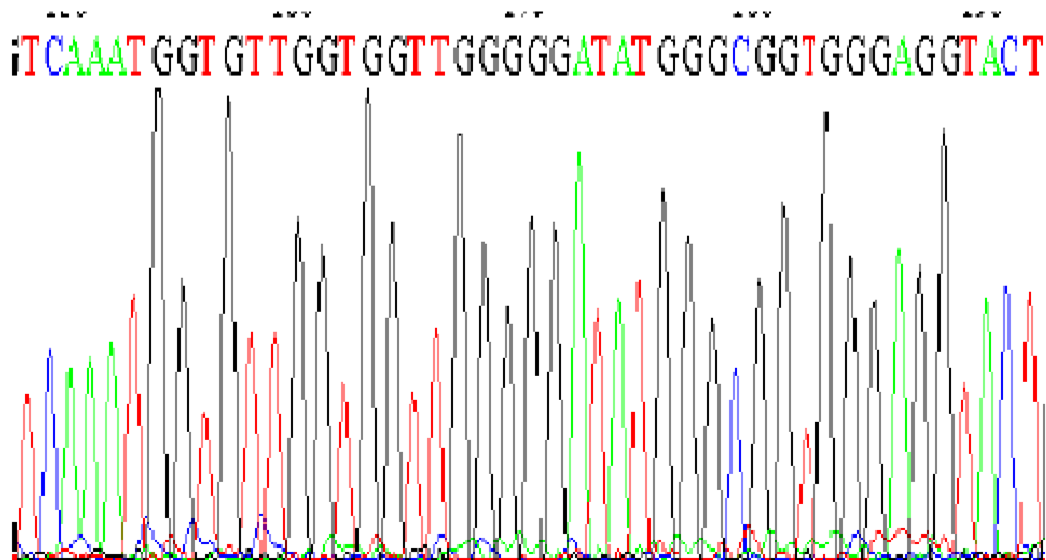


Figure 29. Electropherogram of the random region of anti- Flt3 sequence.

4.6. Investigation for G-rich sequence in the selected aptamer

A web- based server for predicting G- quadruplexes in nucleotide sequences (QGRS mapper) was used to examine our selected- 90 bases anti-Flt3 aptamer for any G-quadruplex, it is available at http://bioinformatics.ramapo.edu/QGRS/analyze.php, and the result is summarized in Table (4).

Table (4). Quadruplex-forming G-rich sequences (QGRS) of anti- Flt3 aptamer using the QGRS mapper.

Position	Length	QGRS	G-Score
21	19	GGTCAAATGGTGTTGGTGG	16
43	28	GGGGATATGGGCGGTGGGAGGTACTGGG	39

Sequence of anti-Flt3 aptamer:

5`GGGATGGATCCAAGCTTACTGG**TCAAATGGTGTTGGTGGTTGGGGGAT ATGGGCGGTGGGAGGTACT**GGGAAGCTTCGATAGGAATTCGG-**3'**

The four groups of (GG) and (GGG) are called the G-tetrads, and the bases in between are called the loops. The G-score was calculated according to the number of Gs in the group and the length of the loop. The higher scores with the most stable G-quadruplexes are related to short loops and high number of G- bases in the guanine tetrads (Kinin, et al., 2006).

There were two QGRS in our selected aptamer; the first was at base number 21, with a G-score equaling 16 and the other was at position 43, with a G-score of 39.

5. Discussion

Nucleic acid aptamers are emerging as a new class of targeted therapeutic agents and are the focus of a rapidly growing field of medical research. These small synthetic oligomers of DNA or RNA form stable three- dimensional structures and bind to defined molecular targets via shape specific recognition. Thus, in essence, their mechanism of action is similar to that of protein- based monoclonal antibodies (Choi, et al, 2009). However, due to their smaller size and different chemical composition, aptamers have a number of potential advantages over monoclonal antibodies. These include ease of manufacture and storage, facile conjugation, better tumor penetration, more rapid systemic clearance and non-immunogenic (Choi, et al, 2009).

The SELEX process is a versatile method for identifying nucleic acids that bind to a variety of molecular entities with high affinity and specificity. The aim of the SELEX experiment described here is to identify ssDNA aptamers against the active tyrosine kinase domain of both, the normal and mutant form of Flt-3 enzyme. This enzyme confers proliferative and anti-apoptotic effects on normal and leukemic hematopoietic stem cells (Scholl, et al., 2005). Mutations in the Flt3 receptor represent the most common known genetic alteration in AML blasts. These mutations cluster in 2 different regions of Flt3; the juxtamembrane region and the TKD. The later results mostly in the substitution of tyrosine for aspartic acid at position 835 within the activation loop (D835Y) causing constitutive activation of the receptor (Choudhary, et al., 2005).

ssDNA fragments of the synthetic library were 90 bases in length**,** with 45 nucleotides random region flanked by defined sequences of the forward and reverse primers, and restriction sites for BamHI, HindIII and EcoRI restriction enzymes. This library was designed to give theoretically 4^{45} different sequences, (where 4 is the number of

nitrogenous bases in the random region), which represents about 1.24×10^{27} different copies.

Synthesis of the first ssDNA library was performed under the same conditions that were used during the trial extension reactions to ensure that the final pool has a complexity similar to the calculated one. In other words, when the small-scale PCR reaction is optimized by using one hundred nanograms of the initial template which represents about 1×10^{12} different sequences in 50 µl of total volume PCR reaction, it was possible to amplify 10µg of synthesized templates (approximately 1×10^{14} different sequences) in 5000 µl of reaction mix. Similar approaches were considered for asymmetric PCR.

Recovery of ssDNA from asymmetric PCR product was performed by extraction and purification from low melting point agarose gel. This method showed good yields of ssDNA after extraction, enough to recover the largest amount of amplified ssDNA with over than 90% as described by Kurien and Scofield (2002).

This study describes a method for selection of ssDNA aptamers against GST tagged kinase domain of Flt-3 enzyme using µMacs magnetic beads, as affinity separation method of aptamer-target complex.

After cloning and sequencing the successfully cloned plasmid, one sequence of anti-Flt3 aptamer was obtained. It is obvious, according to Table (3) listed previously, that the most frequent base with the highest percentage (43%) is guanine (G). The secondary structure of anti-Flt-3 aptamer was predicted by Mfold program as described by Michael Zuker which is available at http://www.bioinfo.rpi.edu/applications/mfold/ (Zuker, 2003). It is the recommended program in the literature because it takes into consideration the folding and binding conditions of aptamers such as temperature and ionic strength which affect the three dimensional structure of the aptamer. Eleven possible secondary structures were predicted for the resulted aptamer. Free energy

reflects the stability of the folding structure of the aptamer, the smallest free energy indicating that the folded structure is more stable (Zuker, 2003).

The resulted anti-Flt-3 aptamer was investigated for having G- rich motifs within its sequence, two sites that are thought to form G-quartets were found, the first was at position 21 (composed of 19 nucleotides) and the other at position 43 (composed of 28 nucleotides), having G- scores of 16 and 39, respectively, (the higher the G- score, the more stable the structure).

Moreover, our selected aptamer was investigated for having sequence homology with the anti proliferative sequence of the guanine rich oligonucleotide AS1411:

5-GGTGGTGGTGGTTGTGGTGGTGGTGG-3

Fortunately, our anti- Flt-3 aptamer appeared to have this G-rich motif within its sequence:

5\`GGGATGGATCCAAGCTTACTGG**TCAAATGGTGTTGGTGGTTGGGGG ATATGGGCGGTGGGAGGTACT**GGGAAGCTTCGATAGGAATTCGG-**3'**

This means that our selected aptamer may have a promising anti- leukemic effect, comparable to that of AS1411, on the leukemic cells of AML patients, because of the presence of the GGT motif, which is responsible for the anti- proliferative activity on cancerous cells.

This result may be considered significant concerning AML patients, since the mutation type of this enzyme, to which we have selected the aptamer, contributes to about 7% of AML patients.

6. Conclusion

In conclusion, this thesis has described the role and importance of the tyrosine kinase enzyme Flt3 and its mutant form D835Y in AML, and it is the first study that describes the selection of anti- Flt3 aptamer using the SELEX method. Out of 10^{14} different sequences, only one aptamer was isolated. Moreover, G-rich motifs were observed in two positions of the selected aptamer, and there were sequence homology between our aptamer and the anti proliferative sequence of AS1411. This may have significant therapeutic and diagnostic importance in the future concerning AML patients.

7. Recommendations

On the basis of the conclusions of this study, the followings are recommended:

- In vitro kinase inhibitory assay performance, to evaluate the inhibitory effect of the selected aptamer against the kinase domain of Flt-3 enzyme.
- Enhance cellular delivery of the selected aptamer, which is an important functional aspect for therapeutic purposes.
- Investigate the effect of G- quadruplex formed by the selected aptamer on the survival of AML leukemic cells.
- Increase nuclease resistance of the selected aptamer.
- Evaluation of the efficiency of inhibition of anti-Flt3 aptamers in vivo.
- Evaluation of the degree of affinity of the selected aptamer for Flt3 active kinase domain.

8. References

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انتقاء سلاسل من الحمض النووي مضادة للإنظيم (Flt3) بطريقة التطوير النظامي لرابطات بالتكاثر المتضاعف

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ملخص

تعد اساليب العلاج التقليدية للأورام السرطانية ذات آثار جانبية عالية وفعالية محدودة في علاج هذه الأورام, مما
أدى إلى التفكير بجيل جديد من العلاجات التي تستهدف الخلايا المصابة دون السليمة و تكون ذات آثار جانبية ضئيلة وذلك اعتماداً على التطور المتسارع في علم البيولوجبا الجزيئية.

تم تطوير تكنولوجيا التطوير النظامي لرابطات بالتكاثر المتضاعف (SELEX) لعزل سلاسل من الحمض النووي تسمى" Aptamers" لديها القدرة على الارتباط بخاصية عالية مع الجزيء المستهدف لاستخدامها في مجالات عديدة مثل التشخيص والعلاج.

بناءً على ذلك فإن هذه الدراسة تهدف إلى عزل سلاسل من الحمض النووي وحيد السلسلة (single stranded DNA aptamers) التي ترتبط بالإنظيم (Flt-3) الذي يلعب دوراً مهماً وأساسياً في عملية إنتاج الكرات الدموية مما قد يؤثر بشكل مباشر في الحد من زيادة إنتاج الخلايا السرطانية مما له أهمية علاجية لمرضى الأورام الدموية (AML).